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ASPECTS OF INSECT ENDOCRINOLOGY

Conference Editor, DIETRICH BODENSTEIN

LIST OF AUTHORS

DIETRICH BODENSTEIN AND KARL MARAMOROSCH (*Conference Cochairmen*), F. ENGELMANN, H. LAUFER, M. LÜSCHER, H. PIEPHO, AND D. G. SHAPPIRIO.

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INTRODUCTORY REMARKS

Dietrich Bodenstein
University of Virginia, Charlottesville, Va.

In the earlier days of insect endocrinology we were concerned chiefly with issues that revolved around the problem of the humoral control of growth and metamorphosis. Although this is still a matter of cardinal importance and a problem that is far from settled, the study of insect endocrinology is rapidly expanding, taking into its domain more and more aspects of biology, thus opening new and unexpected vistas to the researcher. A look at our table of contents will convince readers of this.

It is very fortunate that it was possible to include distinguished scientists from abroad and from our own country among the contributors who discuss current advances in insect endocrinology in these pages. These discussions, I hope, will provide a stimulus for further research in this field.

BLOOD PROTEINS IN INSECT DEVELOPMENT*

Hans Laufer†

Department of Biology, The Johns Hopkins University, Baltimore, Md., and Department of Embryology, Carnegie Institution of Washington, Baltimore, Md.

The giant silk moths *Hyalophora cecropia* and *Samia cynthia*, holometabolous insects, pass through a complex developmental cycle. Before reaching adulthood these animals undergo a series of transformations from the egg through five larval instars and a pupal stage. It has been shown by many investigators that the endocrine glands of these animals control all of the postembryonic transformations. Undoubtedly, all of these hormonally initiated alterations are accompanied by a changing protein constitution.

A number of studies (Telfer and Williams, 1953; Telfer, 1954; Laufer, 1959*a*; Denucé, 1958; and others) have shown that blood proteins change during development of the silk moths. Of particular interest were the studies of Telfer, who used immunochemical means to follow the concentrations of seven proteins during development of the pupa and adult. The change in each antigenic protein seemed to be independent of the others, so that one might expect an independent rate of synthesis and utilization as well as function for each constituent.

It is the intent of the present study to describe in some detail, by means of zone electrophoresis in starch gels, the changes that occur in protein constitution during development of *cecropia* and *cynthia*. These changes are related to the activities of specific organs and to the development and endocrinological activities of the organism. Some electrophoretically separated proteins are identified as antigens. The biological activities, for example, the enzymatic activities, of a number of constituents are described.

Methods and Materials

Experimental organisms. The pupae used in this study were supplied by commercial dealers. Developing adults were obtained by breaking diapause in pupae and permitting development to proceed to a desired point. Adults either were mated spontaneously in the laboratory or, if reluctant, were induced to do so. After the eggs hatched, the larvae were reared on food plants covered with nylon or cotton nets;‡ *cecropia* on cherry, *cynthia* on *Ailanthus*.

Preparation of blood samples. Blood was collected under aseptic conditions using sterile instruments from animals that had been sterilized on their surfaces by immersion for approximately three minutes in a mixture of 50 per cent ethanol and 0.05 per cent mercuric chloride, or in 70 per cent ethanol. The animals were then rinsed in distilled water and dried with absorbent

* The work described in this paper was supported in part by Research Grant G-9833 from the National Science Foundation, Washington, D.C.

Preliminary results of this work have been reported to the American Society of Zoologists (Laufer, 1959*a*), and were also published in the *Carnegie Institution of Washington Year Book* **57:** 361–364 (1958) and **58:** 392–394 (1959).

† Fellow of the National Research Council (1957–1959).

‡ The material for these nets was obtained as a gift from the Mount Vernon Mills, Inc., Baltimore, Md.

paper. Samples of blood were obtained from pupae by an abdominal incision. Larvae were bled from an anterior and dorsal cut through the skin and the adults were bled from the neck after decapitation. Blood was extruded from the animals by gentle pressure into test tubes containing a few milligrams of streptomycin and phenylthiourea (PTU); the streptomycin served as an antibiotic and the PTU inhibited tyrosinase activity, therefore preventing blackening of the blood. The solutions were then centrifuged until cleared of cellular material. Samples were used at their original concentration either the same day they were obtained or were frozen at −20° C. until used. No appreciable effect of freezing was observed on the resolution of protein by zone electrophoresis.

Samples were usually prepared from individual animals. Each sample was subjected to electrophoretic analysis by at least two separate insertions in starch. At least three to five animals were analyzed at each stage studied, but many more fifth instars, pupae, and developing adults were examined. Eggs and larvae, up to the third instar, were extracted as pooled samples. The number of specimens used in the individual experiments is cited in the method for the experiment.

Preparation of tissue extracts. Tissues were dissected out of bled animals under aseptic conditions and rinsed in insect Ringer's solution to remove adhering blood; excess fluid was drawn off with filter paper. The tissues were then ground cold with an all-glass homogenizer, usually without additional fluid. PTU and streptomycin were always added. A cleared supernatant obtained by centrifugation was then assayed in the same manner as the blood samples. The number of extracts prepared from each tissue varied from one to five; each extract was analyzed at least twice.

Protein concentrations were determined by the biuret reaction performed on blood and tissue samples according to the method of Gornall *et al.* (1949).

Electrophoresis in starch gels. Zone electrophoresis was carried out in starch gels according to the method of Smithies (1955), with some modifications. Hydrolyzed potato starch (15 per cent) was used in barbital buffer (*p*H 8.6, 0.02 *M* at 4° C.). The gels possessed resolving properties similar to the starch provided by the Connaught Medical Research Laboratories of the University of Toronto, Toronto, Canada. Measured quantities of protein samples were inserted into the starch on slips of Whatman No. 1 filter paper. Held in the horizontal plane, the gels were subjected either to a constant current of 5 mAmp. for each tray or a constant voltage drop of 3.5 v/cm. Electrophoresis was continued for 16 hours, resulting in the resolution of several components. Both conditions of the current were equally satisfactory and produced similar results.

Following the application of the electrophoretic current, the starch strips were sectioned horizontally with a thin wire into two equal 3-mm. thick strips. These could be halved again, this time by a vertical cut, resulting in four equal segments all containing the same patterns. Adjacent segments containing the same proteins were then stained by a number of procedures and the resulting patterns were compared.

Protein staining. Visualization of the components resolved was accom-

plished by one of two general procedures, one for protein and the other for enzymes. Using one half of the starch strips, proteins were stained by the procedure of Smithies (1955) with a saturated solution of Buffalo Black NBR (Naphthol Blue Black) in 5 parts methanol, 5 parts water and, 1 part glacial acetic acid (Smithies' solution). Greatest detail was obtained by staining for 30 min., followed by destaining of the starch with several prolonged rinses in Smithies' solution without the dye.

Histochemical procedures. The remaining halves of the starch strips, those not stained for protein, were tested for enzymatic activity by histochemical procedures. The methods used for esterase were essentially those described by Hunter and Markert (1957). Esterase substrates that produced positive results when tested with *cecropia* or *cynthia* blood included the following compounds: α-naphthyl acetate, β-naphthyl acetate, α-naphthyl butyrate,* β-naphthyl propionate,† β-naphthyl caprylate,† β-naphthyl laurate, and indoxyl butyrate.‡ Starch strips were incubated in 2 ml. of a 1 per cent acetone solution of substrate in 98 ml. *p*H 6.8 phosphate buffer for 30 min. to 3 hours, depending on the staining intensity of the reaction being studied as determined by preliminary tests. About 100 mg. of the diazonium salt, fast blue RR or fast blue BB,§ were added in 10 ml. to 100 ml. of the incubating substrate-buffer mixture. Naphthol was released by hydrolysis of the substrate. The dye salt coupled with the naphthol, in a diazotization reaction, producing insoluble pigment at the site of enzyme activity. Eserine, in a final concentration of 1×10^{-4} *M*, was applied to the starch strips 30 min. before the addition of substrate as an esterase inhibitor.

To demonstrate other hydrolytic enzyme activities only the substrates were usually changed. The reactions were otherwise carried out in the same manner as those used to indicate esterase activity. The *p*H was altered from 6.8 only in a few experiments. The reactions for alkaline phosphatase were maintained in buffer at a *p*H of 8.6. The phosphatase substrates were sodium alpha-naphthyl acid phosphate and sodium beta-naphthyl acid phosphate; for sulfatase, the substrate was potassium 6-bromo-2-naphthyl sulfate. For chymotrypsin, N-benzoyl-DL-phenylalanine beta-naphthol ester served as the substrate; 6-bromo-2-naphthyl beta-D-glucopyranoside and 6-bromo-2-naphthyl beta-D-galactopyranoside were used for gluco- and galactosidase activity, respectively. Tyrosinase on the starch strips was demonstrated by the addition of 0.1 gm. dihydroxy-phenylalanine (Dopa) to an incubation mixture buffered at *p*H 6.8.

Immunochemical procedures. Antibodies to blood from diapausing *cecropia* pupae were produced in rabbits by the immunization procedure of Telfer (1954). These were used to test the antigenicity and immunological specificity of insect blood proteins. Analyses of the antigen and antibodies were carried out by a modification of the Ouchterlony double agar diffusion method (Laufer, 1959*b*).

* Donated by R. L. Hunter and C. L. Markert.

† These substrates were supplied by Arnold Seligman.

‡ Indoxyl substrates required incubation in buffer only, and no diazonium salts were added in these reactions. Supplied by Arnold Seligman.

§ Borden Chemical Co., Dajac Division, Philadelphia, Pa.

Fractions were prepared from *cecropia* blood by the use of gradient elution ion exchange chromatography according to the method of Sober *et al.* (1956), and analyzed by electrophoresis and by means of immunochemistry for purity, specificity, and enzymatic activity. The immune precipitate bands in agar diffusion plates were also stained directly by histochemical procedures.

Regeneration of blood proteins. An experimental series was prepared to analyze the capacity of the diapausing pupa to reconstitute its blood components after dilution. There were two controls: (1) animals with 0.1 cc. of blood removed; and (2) animals with about 1.0 cc. of blood removed and 0.9 cc. replaced by insect blood. The experimental group was composed of pupae that also had a large sample of blood replaced, this time by Ringer's solution. PTU and streptomycin were added to the wounds, which were then sealed by a plastic window attached to the cuticle with molten paraffin. The blood and Ringer's solution of the experimental organisms were permitted to mix for two hours. After that time the blood was again sampled from a small hole in the window. Four days after these bleedings the blood was sampled again. The different aliquots from one animal were then analyzed by electrophoresis and compared. The following points were examined: (1) the appearance of the normal patterns of proteins and enzymes in the original sample; (2) the amount of dilution of the blood proteins in the animals filled with Ringer's solution; and (3) comparison of all the samples for changes in protein constitution, including regeneration.

Midgut removal from pupae. The role that the midgut plays in regeneration of blood proteins was tested by the removal of this organ. First the animals were bled and then the gut was removed through the same incision. The midgut is a solid rod in *cynthia* and could be extruded intact by the application of sufficient pressure. Following the gut removal, blood or saline was placed in the body cavity. PTU and streptomycin were added to the wound opening, which was then sealed by cementing a coverslip to the cuticle with molten paraffin. A total of 12 diapausing pupae were prepared free of their midgut. Seven animals also had their blood diluted with saline. As one control the blood of 10 normal diapausing pupae was studied. Also examined was the blood of 10 animals in which blood had been diluted with saline but in which the gut had not been removed.

Brain removal from larvae. To determine whether the brain influences the concentration of blood proteins, the brain was successfully extirpated from seven fifth-instar larvae at the time of major protein increase. Four others were wounded in the brain region without removal of the brain, and in three the brain was removed and then returned to its position. The operation was performed through a small incision in the soft neck integument at the dorsal midline behind the head capsule by cutting the lateral connections while holding the brain with watchmaker's forceps. PTU and streptomycin were added to the opening. The wound was closed with molten paraffin.

Results

Proteins in cecropia blood. Electrophoresis followed by staining for protein with Naphthol Blue Black reveals a series of protein bands in the blood

of *cecropia* of varying intensities, which appear to change in number as well as in concentration, or intensity, throughout the life cycle (FIGURE 1). For convenience the four major fractions have been numbered 1 through 4, starting with the designation of 1 for the most rapidly migrating component. Traces of

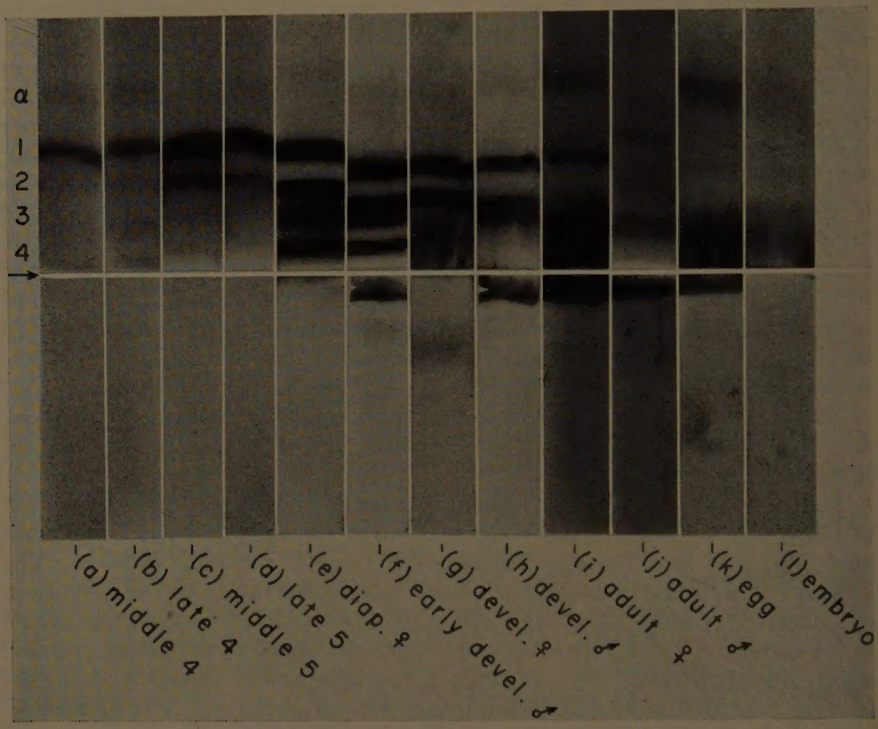

FIGURE 1. *Cecropia* and *cynthia* hemolymph proteins are depicted in this and the succeeding three figures as they were separated by zone electrophoresis in starch gels. The gels were stained either for protein or for enzymes by histochemical procedures. Electrophoresis was carried out for 16 hours at 4° C., *p*H 8.6 in 0.02 *M* barbital buffer. Samples were applied at the point indicated by the arrow; the anode is toward the top of the figure.

The illustration represents the patterns of blood proteins during the life cycle of *cecropia*. Blood from a diapausing female appears to have the highest concentration of the four major proteins. The number and intensity of the components appears to change markedly throughout the life cycle.

other minor constituents are also present as part of the patterns, indicated by the fainter bands at certain stages. However these components are not always reliably reproduced, probably because they lie at the limit of sensitivity of the protein-staining procedure. Some of these are detectable with enzyme reactions, as shown in subsequent sections.

It is clear that protein 1 is the predominant protein in all stages except in mature adults and in the egg. Proteins 2, 3, and 4 have a more transient existence in the blood. The concentration change of each can be followed through several stages of development. Correspondence of similar bands is clear

despite the fact that in these experminents the migration rate was not entirely uniform from one experiment to the next. However, when samples are run side-by-side simultaneously in the same starch gel, the correspondence of components is always precise.

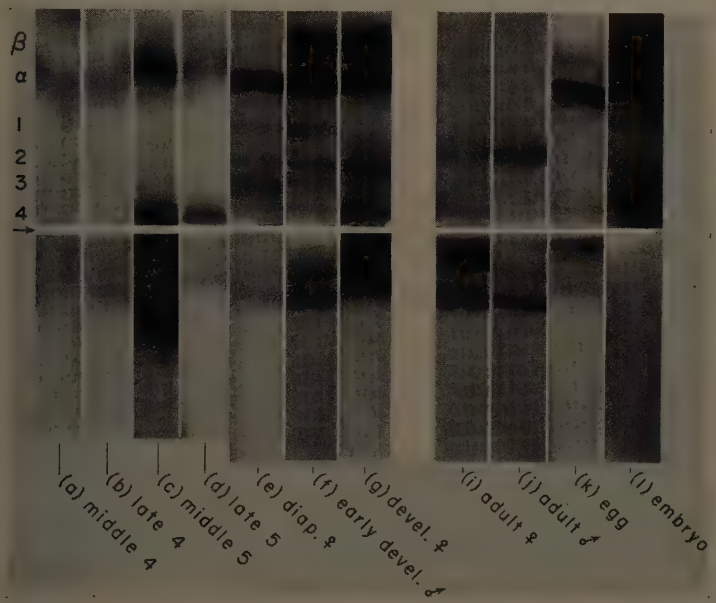

FIGURE 2. Using the procedure described for FIGURE 1, the patterns shown represent the esterase activity of the blood proteins as revealed by the substrate α-naphthyl butyrate during the life cycle of *cecropia*. The starch gels shown here were taken from the same strips as those in FIGURE 1, and the letters and stages of this figure are also comparable. Many of the major protein bands appear to possess esterase activity.

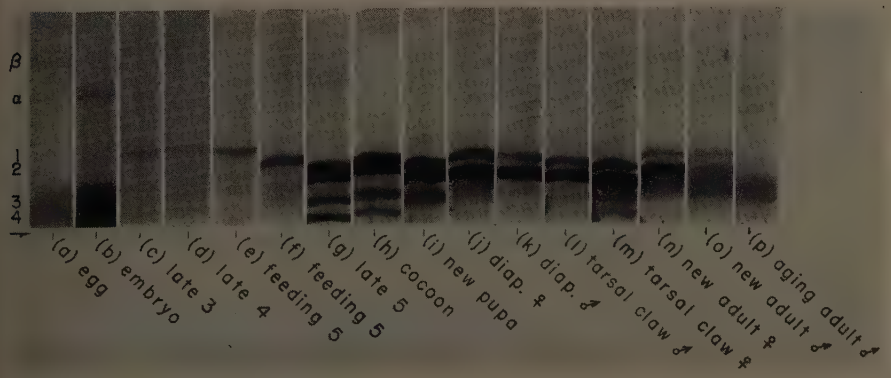

FIGURE 3. Under the same conditions described for FIGURE 1, the patterns shown represent the blood proteins during the life cycle of *cynthia* as they were resolved by starch gel electrophoresis.

The highest concentration of proteins corresponding to the most intense stain uptake in electrophoresis in *cecropia* blood is in the pupa. The major protein components decrease in number and staining intensity with the maturation of the adult. Some may persist in all stages in low concentration. Note that certain blood proteins appear to be directly incorporated into the egg, and thus exhibit behavior similar to that of antigen 7, described by Telfer (1954). Not only can each phase of the life cycle be characterized by definite

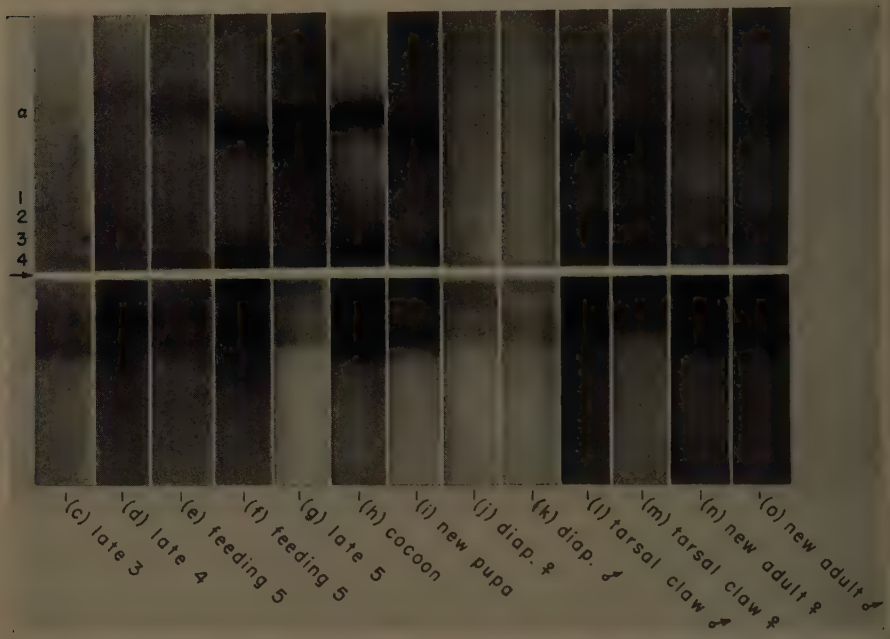

FIGURE 4. The patterns represent the esterase activity of the blood proteins, as revealed by the substrate α-naphthyl butyrate, during the life cycle of *cynthia*. The procedure of this study was that described for FIGURE 1. The starch gels of this figure were taken from the same starch gels as those shown in FIGURE 3, and the letters and stages of life also correspond. Only a few patterns have been omitted. Note especially the low activity of the positively migrating esterases in the blood of diapausing animals. The esterase migrating rapidly toward the anode is inhibited by 1×10^{-4} *M* eserine.

and specific patterns of protein bands but the blood of pupae and later stages can be used to distinguish the sex of the animal from which it was derived. Blood from female pupae possesses a band in the region of protein 3 that is wider than the comparable component in the males (FIGURE 1*e* and *f*). This indicates a higher concentration of one or more proteins in the blood of females.

Proteins in cynthia blood. The pattern of proteins, particularly the four major ones in the development of *cynthia*, appear generally to be similar to *cecropia*, with some variations. Protein 1 occurs in relatively low concentrations in the third to fifth larval stages (FIGURE 3*c*, *d*, *e*, and *f*), and apparently increases in the fifth instar; proteins 2, 3, and 4 increase more

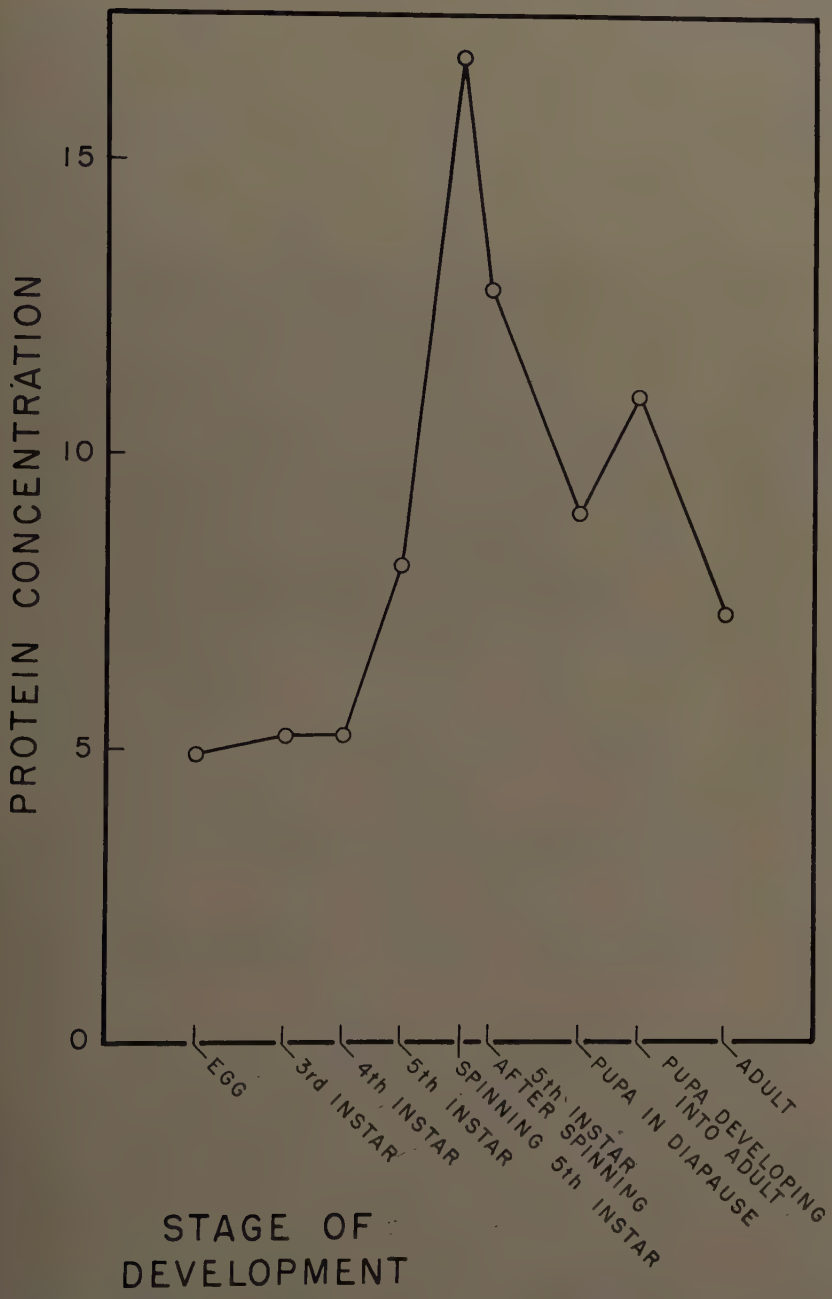

FIGURE 5. The relative concentration of blood protein in *cynthia* during several stages of development.

sharply in the later phases of the last instar stages (FIGURE 3*g*). Whereas four major components persisted throughout pupal diapause in *cecropia*, only two persist in *cynthia* (FIGURE 3*h*, *i*, *j*, and *k*). In *cynthia* as in *cecropia*, an additional protein exists in much higher concentration in females than in males during diapause. While the over-all concentration of proteins decreases during development into the adult, as it does in *cecropia*, several bands persist to the time of emergence of the adult. After emergence, however, the con-

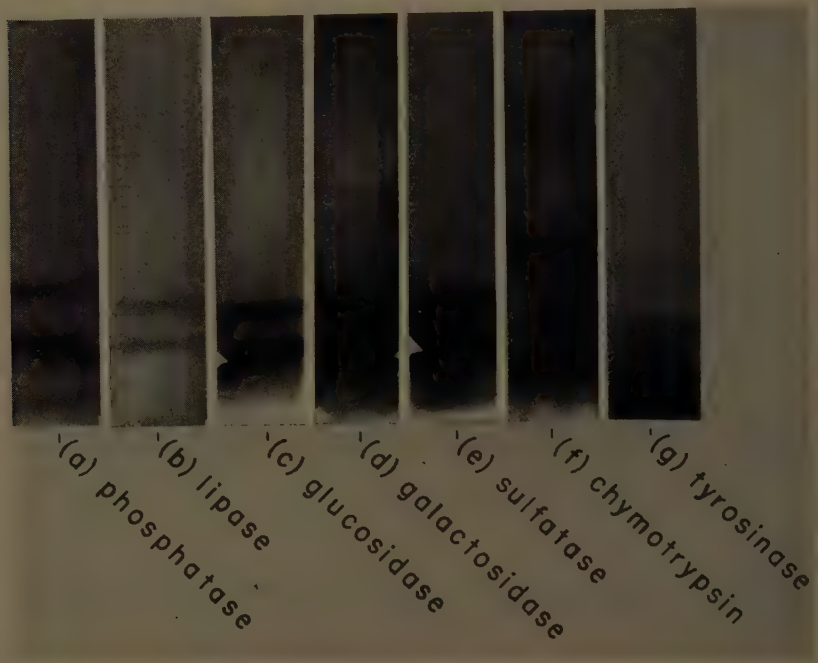

FIGURE 6. Enzymatic activities (zymograms) of *cecropia* blood. Patterns *a* through *e* were obtained from the same sample of diapausing female blood, while patterns *f* and *g* were prepared from a developing female. A variety of enzymatic activities is observed in the proteins of the blood.

centration of protein components of the individual bands declines to a considerable extent (FIGURE 3*l*, *m*, *n*, *o*, and *p*). The highest concentration of protein was found in the spinning fifth instar larva, based on optical readings using the biuret reaction with no reference to absolute values (FIGURE 5). This high level falls with diapause, to rise again somewhat during development of the adult. The protein content of the blood decreases again in adulthood.

A broad dense band is present in preparations of the egg and embryo (FIGURE 3*a* and *b*), denoting one or more constituents in relatively high concentration. However, protein concentration in the egg extract appears to be somewhat lower than that of the embryos, since one volume of saline was used in extracting the eggs while no fluid was added to the embryos or any of the other samples. The migration and staining properties of this component are

similar to a constituent found in the blood of diapausing female pupae and of developing and adult female moths (FIGURE 3*j*, *m*, and *n*), and probably indicates the transfer of protein across the barriers between the blood stream and the interior of the egg.

Although additional protein bands were observable by protein-staining procedures, they were often only barely detectable and could therefore not be followed reliably by this procedure. The intensity of the patterns obtained by electrophoresis corresponded well with concentration of total protein.

Enzymatic activities detected by starch electrophoresis. Since the proteins appear to vary during development, each independent of the other, it is reasonable to expect each to have an individual function, a place of origin within the organism, and an independence of the factors controlling the rate of synthesis and of utilization. One approach to characterize the functional activity of proteins is by means of studies of enzymatic activity. Histochemical staining of starch gels showed that some of these bands possess a variety of enzymatic activities, including esterases, phosphatases, gluco- and galactosidases, sulfatases, chymotrypsins, and tyrosinases (FIGURE 6). The protein bands that contain most of these activities are proteins 1 and 2 in the diapausing *cecropia* pupa. With development, the spectrum of patterns changes.

Esterases in developing cecropia. The esterase activity of bands resolved by zone electrophoresis changes markedly during development. FIGURE 2 is a photograph of starch strips that are the same as those shown in FIGURE 1. However, these patterns for esterases were obtained by staining blood samples with α-naphthyl butyrate. The greatest activity is found in the early phases of the development of the adult when as many as eight distinct bands are observed (FIGURE 2*f*). It is consistently found that two dense esterase bands and intensification of some of the other components characterized early development into the adult (compare FIGURE 2*e* and *f*). With the progression of development the intensities diminish until in the mature adult only a fraction of the activity remains, some of which is then completely undetectable or greatly decreased. On the other hand, the unfertilized egg has esterases with similar migratory properties to those in the developing adult. The results indicate an incorporation of these proteins into the developing egg. The esterase pattern is altered further in eggs containing maturing embryos.

Individuality of the esterase. The question whether the esterase from *cecropia* blood can be distinguished on the basis of differing substrate specificities and differential enzyme inhibition was considered next. The blood of a developing pupa was resolved by electrophoresis into six positively migrating esterases when incubated with the substrate α-naphthyl butyrate (FIGURE 7*a*). One of the two most active components was inhibited in an adjacent section treated identically with the same substrate but with the addition of eserine to the incubation mixture (FIGURE 7*b*). Eserine inhibited one of the two rapid and most active esterases. It is these two esterases that can utilize indoxyl butyrate (FIGURE 7*c*), while β-naphthyl caprylate is utilized only by the middle two of the six positively migrating esterases (FIGURE 7*d*). The results indicate the existence of considerable substrate- and inhibition-specificities among these esterases. The implication of these results is that these enzymes fall into

specificity groups that differ considerably from each other and metabolize different substrates *in vivo*. However there seem to be groups or classes of esterases that thus far cannot be distinguished from one another within the group, except by electrophoretic mobility. These may be "isozymes," according to the criteria of Markert and Møller (1959).

Esterases in cynthia blood. Change in esterase activity in *cynthia* blood is not as spectacular as it is in *cecropia* blood, for the bands never become as

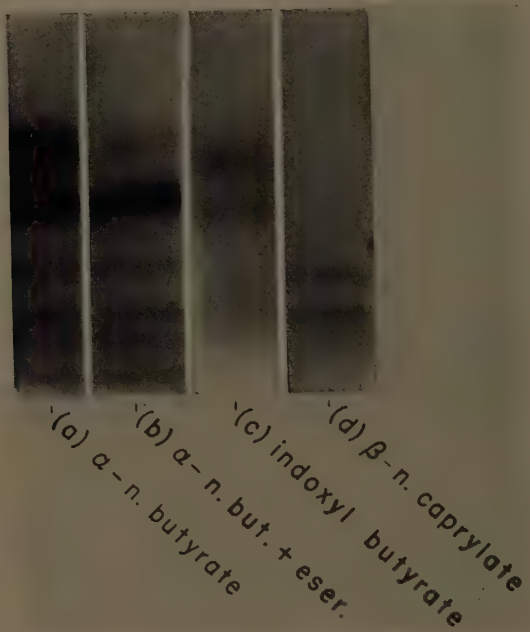

FIGURE 7. Blood from a *cecropia* pupa developing into an adult was tested for esterase activity with several substrates; *a* shows the pattern obtained with α-naphthyl butyrate; *b* indicates the action of α-naphthyl butyrate with 1×10^{-4} *M* of eserine added as an inhibitor. Indoxyl butyrate and β-naphthyl caprylate were used as substrates in *c* and *d*. Most of the esterases are distinguished on the basis of their substrate and inhibitor specificities.

intense. However, the same sort of generalization seems to apply: esterase activity decreases with diapause. The rapidly migrating esterases that are present before and after diapause usually are not observable in the pupa in diapause. With the initiation of development these esterases reappear. This observation of the lack of rapidly migrating esterase activity in diapause has been made in some 50 animals, whereas a group of animals of different developmental stages possessed these enzymatic activities.

Localization of proteins and enzymes within tissues of cynthia. Among the questions of interest in regard to the changes in blood proteins are those concerned with the source and the disposition of these proteins. For example, where are the components synthesized, where are they utilized? The answers to these questions may in time provide clues to further questions, such as what

they do, and what is the functional significance of these proteins. As a standard for comparison, photographs of starch strips containing blood from diapausing animals are included.

FIGURE 8 depicts samples of *cynthia* blood from males and females compared to samples derived from fat body. Each tissue, like the blood, has its own particular pattern of protein bands. Just as the proteins differ in the bloods of the two sexes, so do they differ in their respective fat bodies (compare FIGURE 8*a* and *b* with *c*, *d*, and *e*). Protein 3, then, is found in higher concentration in female blood and fat body than in either blood or fat body obtained from males. Another question that should be touched upon here is the mechanism of the movement of these proteins from the cellular site of their formation to their final position, such as in the egg. Since the high concentration of one of the blood proteins in the egg was shown above, presumably the same one that is found in fat body, the possibility must be entertained that there is passage of protein molecules from fat body to the blood to the egg. For protein to enter the egg proper from the blood as intact molecules, cellular membranes and other barriers must be traversed.

Protein 1 occurs in greater concentration in the fat body than in blood, while protein 2 is present in the fat body in lower concentration than in blood. Protein 4 is present in fat body but absent from blood during diapause. It should be recalled that a component with electrophoretic properties similar to protein 4 is seen during the late fifth instar larva and again during approximately the middle of development of the adult. Such a component was a so seen when the blood proteins were depleted by experimental bleeding (see below).

These observations strongly implicate the fat body as the source of components 1 and 4 and also the female-specific protein.

The protein patterns of other tissues, such as epidermis, silk gland, and gut, are presented for comparison in FIGURE 8 along with the patterns for blood and meconium. Each tissue and fluid appears to have its own specific pattern of proteins, as revealed by zone electrophoresis in the starch gel.

The esterases from various tissues and fluids of the body resolve into distinct patterns of enzymes or zymograms on the starch gels (Markert and Hunter, 1959); furthermore, some of these enzymes appear to be the same as those already seen in the blood. For example, FIGURE 8 shows a comparison of blood and fat body esterases during development and in diapause.

Other tissues, such as silk gland and midgut, also yield characteristic zymograms (FIGURE 8*o*). Midgut preparations are so active that large segments of the strips were blackened by the reaction. Since there was always some overlapping of components these reactions are not depicted. However, the presence of certain esterases in the gut of *cynthia*, as well as in *cecropia*, suggested that the gut is a major determinant in the concentration of these esterases in the blood. Consequently, gut extirpation experiments were initiated that are described below.

Localization of proteins and enzymes within tissues of cecropia. The protein and enzyme content of blood and a number of other tissues were compared to see if some clue could be obtained on the source and disposition of the con-

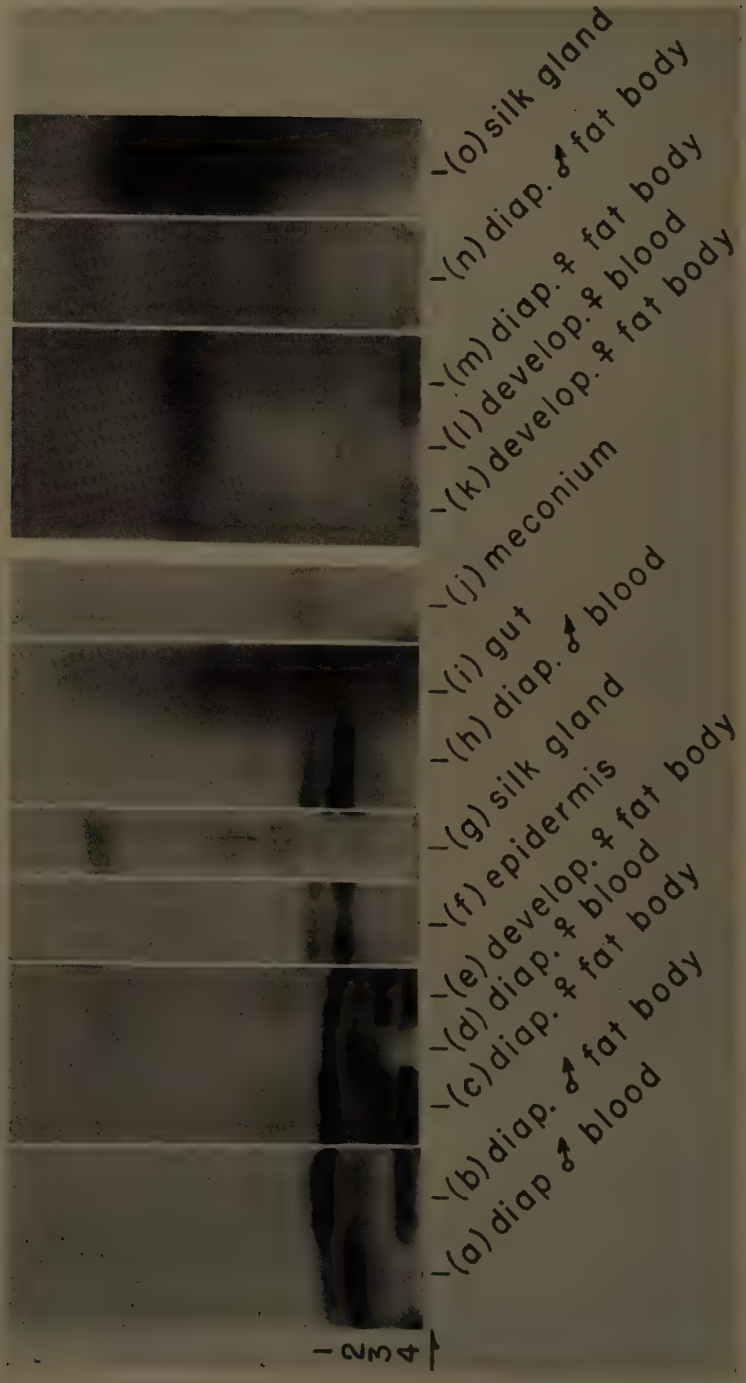

FIGURE 8. The protein patterns and zymograms of several *cynthia* tissues, compared with blood proteins. Each tissue possesses its characteristic patterns of bands, either when stained for protein as in the patterns *a* to *j*, or when stained for esterases with α-naphthyl butyrate, as in *h* to *o*.

stituents. It was immediately apparent that tissues differ greatly from one another with respect to their protein content. The esterase and phosphatase zymograms are also specific for each tissue, as shown in FIGURE 9. The adjacent patterns compare the gut and fat body extracts (FIGURE 9*d* to *l*). It is apparent that each of the three blood samples (FIGURE 9*e*, *h*, and *k*) has a major esterase and several additional minor ones. Though the two depicted fat body preparations differ somewhat from one another (FIGURE 9*g* and *j*), it is quite clear that they do not match the major blood esterase, but do coincide in mobility with the minor ones. The gut possesses a family of esterases

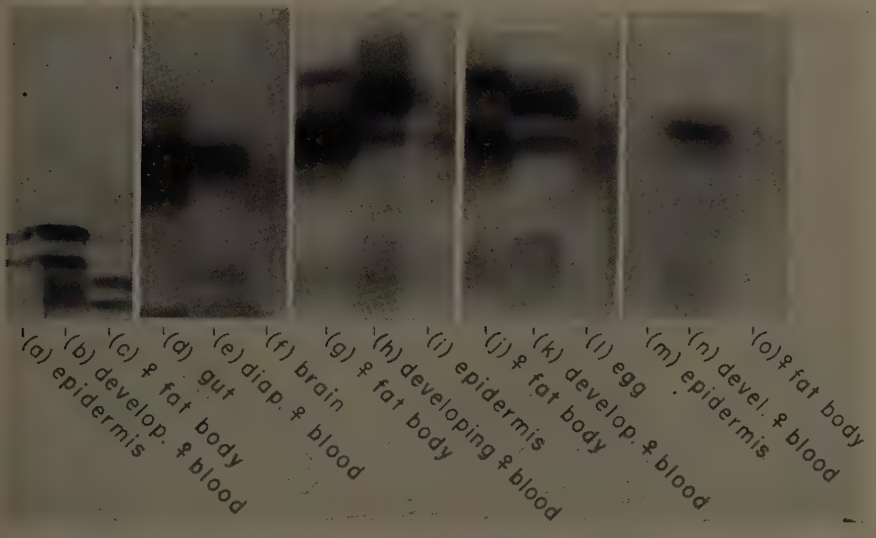

FIGURE 9. The protein patterns (*a* to *c*) and zymograms (*d* to *o*) of several *cecropia* tissues, compared with blood proteins. Each tissue possesses its characteristic pattern of bands. The zymograms were produced with α-naphthyl butyrate as the substrate for esterases (*d* to *l*), while the reactions *m* to *o* are for phosphatases.

of still different composition. Here the major gut esterase appears to coincide in migratory characteristics with the major blood esterase (FIGURE 9*d* and *e*). These findings suggested that this gut esterase might be released into the blood from the gut. This possibility was tested, as described below.

Wounding. It is of some importance in the evaluation of the experimental procedures to find out what effect wounding has on the protein spectrum. The normal protein pattern of a diapausing *cynthia* male, it should be recalled, is composed of two bands (FIGURES 3*k* and 8*a*). The esterase pattern includes relatively weak components, the more prominent ones of which migrate toward the negative electrode (FIGURES 4*k* and 10*a*). If such an organism is injured, as occurs during the withdrawal of a blood sample, increase in esterase activity is observed subsequently among those components migrating in the direction of the negative pole. Such a wounding effect is not only observable in pupae (FIGURE 10*b*) but also in other stages, such as fifth in-

FIGURE 10. (See p. 505.)

star *cynthia* (FIGURE 11*c* and *d*). Similar changes in pattern are also seen in *cecropia*. Therefore, an alteration in pattern should be expected whenever an operation is attempted. Indeed, changes in the blood proteins may be observed within two hours after injury.

The regeneration of blood. When blood is removed from a diapausing pupa and the fluid is replaced by saline, the protein concentration is of course lowered. About 65 per cent of the blood protein content is removed easily, as shown in FIGURE 10*d* and *e*, where the hemolymph sample from a female pupa is compared with a sample taken two hours after dilution of blood by saline. One of these proteins, component 4, is seen in higher concentration in the two-hour sample than is normally found in the blood of the diapausing pupa. Hence, the pupa rapidly regenerates blood proteins. This is borne out by continued observations over longer periods. In four days there is considerable reconstitution of the blood (FIGURE 10*e*). The total protein concentration rises to about 80 per cent of the original. Constituent 4, atypical of this stage, also continues to increase in concentration along with the others, a further striking deviation from the normal blood pattern that occurs during regeneration. Considerable esterase activity appears that indicates migration toward the anode (compare FIGURE 10*c*, *d*, and *e*). These esterases occur in addition to the negatively migrating ones that increased due to wounding. Positively migrating esterases with high activity are also not typical of the diapausing organism. Such esterase activities are found in samples from actively developing stages preceding and following diapause of the pupae.

Gut extirpation. Removal experiments were initiated to determine whether the gut was related to the synthesis of the rapid esterases found to occur in such high concentration in the gut and other tissues and organs. A series of 4 classes of diapausing *cynthia* were prepared: (1) 16 wounded animals that were expected to form "wound esterase" on the basis of experiments described above; (2) 10 animals with blood diluted which, according to earlier results, should form the rapid esterases, as described above; (3) 5 animals with the gut removed and the blood undiluted; and (4) 6 animals with the gut removed and the blood diluted. Each animal was sampled at the time of the operation,

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FIGURE 10. Diapausing *cynthia* pupae operated upon to demonstrate regeneration of blood components and the effect of gut extirpation on regeneration. The upper row of patterns are the positively migrating bands stained for proteins. The lower rows are positively and negatively migrating bands stained for esterases. *a* is blood from a diapausing male pupa used as a normal control. *b* is blood sampled from the same animal, four days later. There is no marked change in the protein pattern, but there is an increased esterase activity after four days that migrates negatively. This increase is often seen as a result of wounding. *c* is blood from a normal diapausing female pupa used as a control. *d* is a sample taken two hours after the blood was diluted with Ringer's solution by more than 50 per cent. *e* is blood from the same animal taken four days later. Not only does the protein concentration increase within four days, but components are present within the blood two hours after dilution that are not normally found at this stage of diapause. The "wound" esterase is present within four days, and so are a number of positively migrating esterases. *f* is blood from a normal diapausing male *cynthia* pupa. *g* is blood sampled two hours after dilution of the pupa blood with saline solution and removal of the gut. *h* is blood from this same animal four days later. The diluted proteins increase in concentration within four days. The wound esterase also increases; signs of its increase are noticeable within two hours. The positively migrating esterases, however, usually found when the gut is intact, are now lacking. This finding implicates the gut as playing a role in the production of blood esterases.

at the beginning of the experiment, and on the fourth day after the operation. The animals with diluted blood were also sampled two hours after the operation to obtain blood after equilibration with saline. The organisms with undiluted blood served as controls. In the wounded animals the activity of negatively migrating components increased according to expectations in all cases (FIGURE 10*a* and *b*, and TABLE 1). Animals with diluted blood and those with the gut removed also showed similar increases. Blood dilution increased the rapid positively migrating esterases as was anticipated (FIGURE 10*c*, *d*, and *e*), but animals with the gut removed and the blood diluted did not show this type of esterase activity (FIGURE 10*f*, *g*, and *h*). The result indicates that the gut is associated with the release and probably the synthesis of particular blood esterases. These esterases are not normally found in the blood of diapausing *cynthia* to any great extent, but are active in the blood during larval and adult development and when the organism is called upon to regenerate lost proteins.

TABLE 1

INCREASED ESTERASE ACTIVITY OF DIAPAUSING *CYNTHIA* PUPAE

Samples Obtained on the Fourth Day are Compared with Those of the First

Treatment	Increased activity in negatively migrating esterases/number of specimens	Increased activity in positively migrating esterases/number of specimens
Wounded; normal blood concentration	16/16	0/16
Gut removed; normal blood concentration	4/5	0/5
Gut retained; blood diluted	7/10	10/10
Gut removed; blood diluted	5/6	0/6

Brain extirpation and arrested development. Experiments were conducted to determine the significance of the brain in the control of the concentration of blood proteins. The blood was sampled in late fifth instar larvae, and samples were also removed thereafter at four-day intervals as long as the animals survived. The brain either was left intact (6 animals), was removed (7 animals), or was severed from its nervous connections (3 animals). The patterns obtained from the normal larvae showed that the original sample either contained four major proteins (FIGURE 11*c* and *d*), or the full complement of four was completed within the next four days (FIGURE 11*a* and *b*). The patterns of protein bands of animals in which the brain was detached from nervous connections were essentially the same as for animals retaining their brains.

In the control animals, the protein complement was usually completed, and the concentration as observed by staining intensity was maintained or increased in the days following the operation (6 of 7 animals). When the brain was extirpated, however, the increase of protein concentration or increase in number of bands was observed in only 1 of 7 animals. The full complement of 4 bands if found originally was either maintained (FIGURE 11*g* and *h*), or the concentration of the proteins present decreased (FIGURE 11*i* and *j*). If the pattern of 4 proteins was not completed (FIGURE 11*e* and *f*) at the begin-

ning, it was usually not completed after brain extirpation, as revealed by subsequent bleedings.

As for the esterase patterns, the fifth instar *cynthia* possessed one active esterase, as many as four minor ones that migrate toward the anode, and also

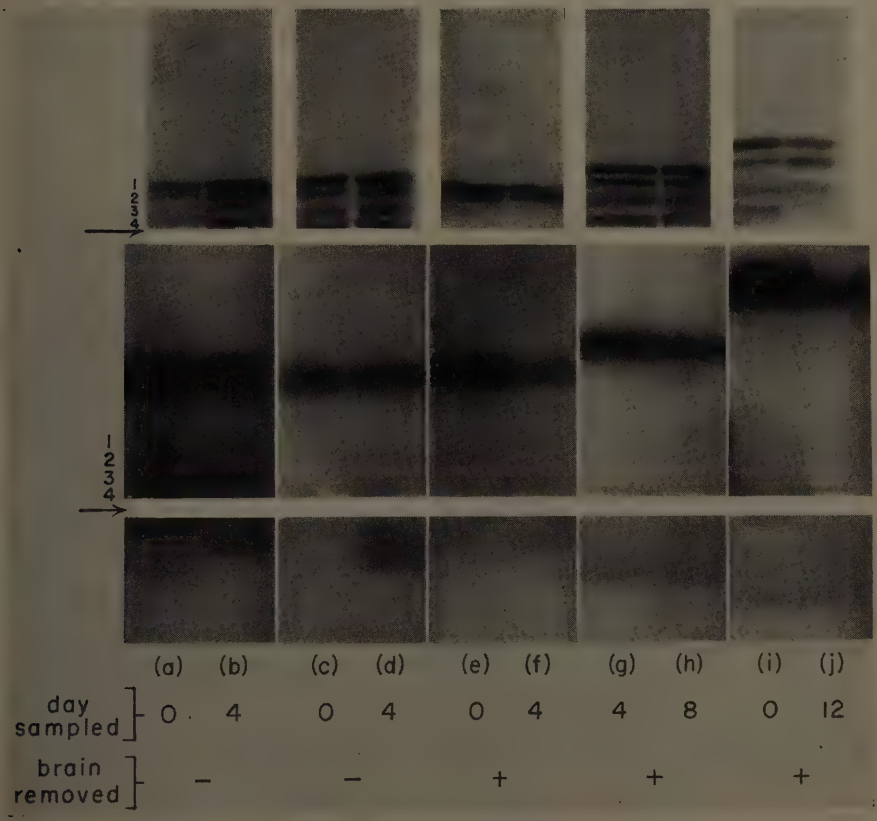

FIGURE 11. The patterns shown are of fifth instar *cynthia* larvae under normal and experimental conditions. The upper row of strips contains the positively migrating patterns stained for protein; those below include both positively and negatively migrating proteins treated to show esterase activity. Note that, in the absence of the brain, proteins do not increase in concentration to a full complement, as they do when the brain is present. Compare especially *a* and *b* with *e* and *f* and with *i* and *j*. Note also the decrease in esterase activity in the "debrained" animals, and the increased negatively migrating esterase activity, presumably due to wounding.

one or more that migrated toward the cathode. Wounding in the larva, as in the pupa, intensified the activity of the negatively migrating esterases. However, when the brain was removed the increased activity was less marked (FIGURE 11*e* to *j*). Even more striking was the loss of esterase activity in the rapid component migrating toward the anode. Some reduction in esterase activity was also observed in larvae when the brain connections were severed but the brain was left in place; however, the reduction in activity was always

more marked when the brain was extirpated. This reduction in activity with cut connections may indicate that the brain's secretory activity is required either directly or indirectly for the maintenance of the enzymes and that intact nervous connections are required for the high activity of the brain. Further studies, however, are necessary to distinguish between these and other possibilities.

Demonstration of identity of antigens and enzymes. Several antigens in *cecropia* blood were examined by Telfer and Williams (1953) and by Telfer (1954), who were able to show that these antigens were proteins and that each of seven different ones changed in concentration independently of the others during

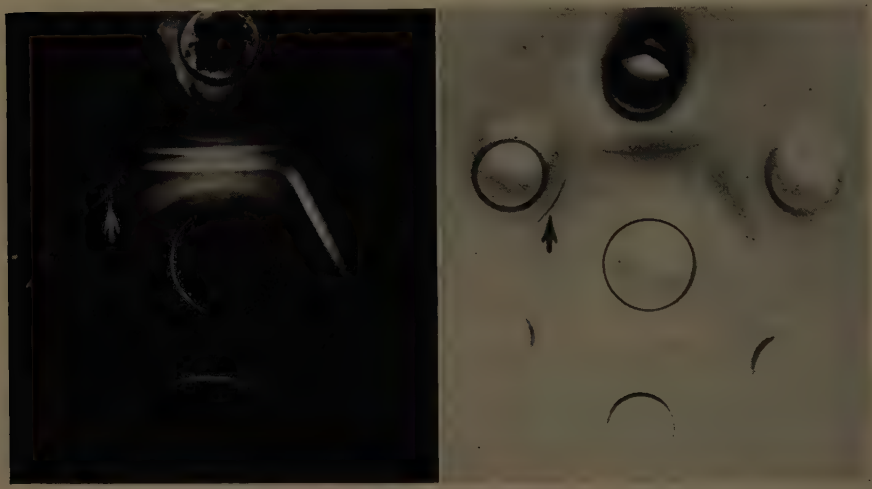

FIGURE 12. Blood from female *cecropia* pupae was examined immunochemically by agar-gel diffusion. The bands of antigen-antibody precipitate show up in the agar as white lines in the photograph at *left*, taken by transmitted light. The entire gel was stained for esterase activity and photographed in direct light. The precipitate bands with esterase activity appear brown in the picture at the *right*. Note particularly the bands with esterase activity of the reaction in the *upper center* of the plate, and the bands to the right and left of it. Electrophoretic analysis shows that the proteins of the upper central reservoir, and those to its right and left, possess esterase activity (*arrows*). By staining the diffusion plate with the same histochemical procedure for esterase as was used for starch, it is clear that there are antigens in this plate that retain their enzymatic activity even in combination with antibody. Thus it is possible to identify directly certain enzymatically active proteins as antigens.

pupation and subsequent development of the adult. While Telfer (1954) demonstrated that one of these proteins was limited to females and was incorporated into eggs against a concentration gradient, no functional significance or biological activity could be ascribed to any of them. Interest therefore lay in the chemical identification of enzymes with antigens and in the examination of the specificity of the proteins. Correlation between electrophoretic bands and antigenic components were made in the course of the present investigation by using immunochemical analyses in agar diffusion (Laufer, 1959*b*). Protein fractions were isolated from insect blood. Each fraction was examined for its electrophoretic and antigenic components. Four major fractions were prepared with gradient elution from DEAE according to the

method of Sober *et al.* (1956). Good correlations between electrophoretic bands and immune precipitates were obtained. A minimum of one precipitate band was observed for each electrophoretic component resolved. In two fractions several immune precipitate bands were formed from each fraction isolated, so that it was necessary to conclude that separation by DEAE is not always complete. Yet positive identification of enzymes with immune bands was possible because direct histochemical treatment of diffusion plates revealed esterase activity despite the fact that the antigens were in combination with antibody. A fraction that contained esterase activity but little protein was analyzed immunochemically and proved to be homogeneous by this criterion. Direct histochemical staining of the agar diffusion plate again confirmed the direct relation of the rapid esterase with a particular antigen (FIGURE 12). A second fraction also retained its esterase activity while in combination with antibody.

Discussion

The procedures used in the present study have great sensitivity and specificity in identifying and characterizing proteins. These proteins have been followed during normal development and the findings indicate that a number of precise changes occur during particular stages of development of *Hyalophora cecropia* and *Samia cynthia*. Experimental procedures have also been initiated to determine the significance of the proteins in development. Before discussing these proteins and their biological significance further, several general problems should be considered. These concern the procedure of starch gel electrophoresis.

A number of enzymatically active bands were resolved from protein mixtures that react with the same substrates; for example, eight esterase bands were observed with α-naphthyl butyrate, two with indoxyl butyrate, two with β-naphthyl caprylate, and three with sodium α-naphthyl acid phosphate. In the case of the eight esterases, most of them have been clearly demonstrated to possess substrate specificities differing from the others. Choline esterase, lipases, and other esterases have been distinguished. Recently Van Asperen (1959) obtained evidence for two and possibly a third esterase by an indirect method of examination of total homogenates of horsefly heads, thoraces, and abdomens. On the basis of electrophoretic evidence one can conclude that at least four distinguishable, and possibly other, types of esterases exist in *cecropia*. However the reactions must be clarified further because they include active bands for the same substrate that have not, thus far, been distinguished from others. It may be, furthermore, that such enzymes will be shown in future experiments to differ in respects other than electrophoretic mobility. On the other hand, it is possible that these proteins differ only slightly in chemical constitution and represent populations with similar or even identical biological activity. In the latter case the proteins would fit into the category of isozymes as defined by Markert and Møller (1959). The nature of the difference of these proteins is yet to be elaborated. It is not uncommon to find a major protein band; for example, the proteins 1 and 2 of *cecropia* or *cynthia*, with esterase, phosphatase, sulfatase, carbohydrase, and chymotryptic activity. In such cases one is faced with the alternatives of a complex family of

proteins with the same electrophoretic mobility or with one or more proteins with broad enzymatic specificity. Perhaps both possibilities hold, for immunochemical analyses have shown that wherever multiple substrates were hydrolyzed in the starch, a number of proteins were found. However, the number of immunochemical components in one electrophoretic band was not very large. On the other hand, the example of chymotrypsin argues for a broad substrate specificity. This enzyme has been shown by Neurath and Hartley (1959), and others, to hydrolyze not only peptide bonds and esters of fatty acids similar to those tested here, but also phosphate esters. It is possible, therefore, that one protein can react with a number of the substrates tested.

Changes in blood proteins. Changes in the number and concentration of blood protein components were demonstrated to occur during the development of both species of silk moths. The study of these proteins and their changes is of particular interest for several reasons. First, the changes observed are signs of the differentiation of the developing organism. This is supported by the finding that the components changed progressively throughout the larval-pupal-adult transformations, but not during the larval molt cycles, during which differentiation is only slight. The alterations in the protein and enzyme spectra were precise and coincided with stages undergoing marked development. Cyclical changes not correlated with development were not observed. The alterations that were found usually consisted of an appearance, decline, and eclipse of one component independent of changes occurring in other proteins. Similar changes in protein pattern were observed by Telfer, who examined *cecropia* hemolymph with immunochemical procedures. Many points of similarity exist between the behavior of the proteins discussed in this paper and Telfer's antigens. More recently Chen (1959) recorded several proteins by starch electrophoresis of *Drosophila* hemolymph. These became more numerous and increased in concentration between the three stages observed. Starch gel electrophoresis has also been applied to *Bombyx* hemolymph by Denucé (1958). A number of points of similarity also exist between the proteins of *Bombyx* and those of *cecropia* and *cynthia*. According to Buck (1953), unpublished experiments of Williams and his co-workers have indicated the presence of six electrophoretic components in *cecropia* pupa blood. The molecular weight of the largest component was 450,000. This protein constituted the highest concentration and was also the most rapidly migrating component. It may, therefore, correspond to protein 1 of the present investigation.

These studies point out the need for further chemical identification of hemolymph proteins as well as the requirement for investigations of their nature, the factors controlling their concentration, synthesis within the organism, and possible biological functions. It should also be mentioned that the concentration changes of proteins in insects are either indirectly the result of hormone action or are the endocrine secretions themselves. Both of these possibilities may be true. Williams (1951) has suggested that the prothoracic gland hormone may correspond to or be associated with a protein fraction.

Enzyme activity in the blood. The finding of a diversity of hydrolytic enzyme activities suggests a possible function for some of the blood proteins. Their

function may also have developmental significance since the activity of certain hydrolytic enzymes coincides with periods of greatest developmental change, particularly when tissues are being lysed or digested for replacement by structures of a subsequent stage. Furthermore, during diapause certain esterases can be stimulated to activity by wounding, while others are activated during blood protein regeneration. The esterases assume additional importance since certain insecticides are presumed to act on one or more of these enzymes (Winteringham and Lewis, 1959). Van der Kloot (1955) has suggested that acetylcholine esterase in the brain may be intimately related to the onset of neurosecretion and the initiation of development of the diapausing pupa into the adult.

It is premature to attempt to ascribe natural substrates to any of the enzymes found by starch electrophoresis, but a number of obvious compounds present themselves. Recent analyses of insect blood suggest possibilities for substrates for the variety of enzymes resolved. For example, phosphate compounds in *cecropia* blood (Wyatt, 1959) include glucose phosphates, adenosine triphosphate (ATP), α-glycerophosphate, phosphorylcholine, phosphoethanolamine, and sorbitol-6-phosphate, among others. Trehalose and sucrose may be substrates for the carbohydrases, while the esterases probably split a great variety of long chain (lipases) and short chain (esterases) fatty acid esters (Niemierko, 1959), and thus may play an important role in lipid metabolism in addition to metabolizing choline esters. It also seems premature to identify enzymes in the present investigation with others reported in the literature.

The possibility has been suggested several times (see, for example, Markert, 1958) that substrates induce enzyme formation during embryonic and postembryonic development, and in this way initiate differentiation. The requirement for the initiation of development in the diapausing silk moth pupa, for example, may be the release of an enzyme that hydrolyzes substrates in stored form. When the concentration of a particular substrate is sufficiently high, synthesis of the corresponding enzyme may be induced. With the accumulation of additional metabolic products, further inductions of enzymes become possible or cellular differentiation results. Proof of such a mechanism during differentiation has thus far not been obtained; however, there are three suggestions for its existence. One is my finding of the esterase activities during diapause and during development. A second is the discovery by Van der Kloot of an increase of cholinergic material in the brain before the onset of neurosecretion, at a time when cholinesterase and electrical activity in the brain were also lacking (see also Monro, 1958). Another example is the accumulation of high levels of amino acids in insect blood that precedes increase in concentration of blood proteins. Such correlation of amino acid increase before significant development has been made in the past (see, for example, Chen, 1956).

In this connection it should be pointed out that certain esterases may be capable of hydrolyzing proteins by splitting peptide bonds. The reverse situation, of proteolytic enzymes possessing esterolytic activity, is known; one example of this type is chymotrypsin (Neurath and Hartley, 1959). Two enzymes found in the present study appear to possess chymotryptic activity, so that it may be appropriate to suggest that some of the blood esterases func-

tion in proteolytic activities during development. In any case it was interesting to observe that the hydrolytic enzymes of the blood appeared to be most active at the time when larval and pupal structures, such as the fat body and certain muscles, were being lysed. This tissue breakdown is a normal part of the developmental sequence.

Gut homogenates of *cecropia* and *cynthia* were particularly active for certain esterases, which corresponded to esterases in the blood. This observation prompted the investigation of this organ as a source of one or more esterases. The simplest interpretation consistent with the results is that the gut does indeed synthesize one or more of these enzymes, since its extirpation decreased the levels of positively migrating blood esterases in every case. In the light of the comments above concerning esterase activity in development, it is interesting to note that Thompson and Møller (1959) suggested that a relationship exists between neurosecretion of the brain and proteinase activity of the gut.

Some general considerations. The information obtained from the present experiments may contribute to the understanding of hormone action. First, it should be noted that the increase of the major proteins during development tends to parallel the action of the prothoracic gland at a time when there is a decline in titer of allatum or juvenile hormone. Furthermore, our preliminary results with the removal of the brain of fifth instar larvae, which resulted in the arrest of development and prevention of the normal increase in protein concentration, may be attributed to a lack of prothoracic gland hormone. However, Fukuda (1944) and others have shown that the brain is required for pupation, but only up to a critical period and not afterwards. A difference exists between these experiments and ours, in that we withdrew blood and continued to injure the organisms on subsequent occasions following brain extirpation. While this did not interfere with development of the sham operated or normal controls, the additional removal of blood, and possibly hormones, was probably sufficient to prevent development, particularly when the brain was no longer present for further prothoracic gland stimulation.

Further experiments may also contribute to information on gene action. Mutants of silk moths are available for genetic and biochemical analyses using procedures as outlined above. Biochemical mutants are known for *Bombyx* (see, for example, the review by Kikkawa, 1953) and for *Drosophila* (see Hadorn, 1958). It is entirely conceivable that in the future phenocopies of known mutants will be induced by surgical procedures and may be shown to be similar or even identical with respect to protein metabolism by a variety of techniques. A suggestion that this is a reasonable approach was prompted by an examination of a *cynthia* larva that had stopped spinning before completing its cocoon. Instead of the normal protein pattern for this stage of four bands, the individual had the protein configuration of an animal with its brain removed. The genetic basis for this defect was, of course, not known, but appropriate mutant stocks may be analyzed in a similar fashion. Pathological conditions due to either natural infections or toxic substances, as, for example, insecticides, would in all probability also reflect themselves in the protein and enzyme spectrum.

Wounding appears to be an interesting tool for the study of development in the diapausing pupa since a number of changes occur as a result of injury. Several changes are observed that resemble those appearing in early development, such as an increase in metabolic rate (Kurland and Schneiderman, 1959) and in synthesis of certain enzymes of the cytochrome system (discussed elsewhere in this monograph by Shappirio). However, the fundamental difference between phenomena of injury and initiation of development is that, following recovery from injury, the diapausing pupa reverts to a state of diapause. The changes that follow wounding appear to be associated with development as well, and may, therefore, be necessary for development. These alterations in proteins can, however, be ruled out as being sufficient cause for development. The other changes accompanying development appear to be unique to this process and may be of fundamental importance.

Summary

This paper deals with the changes in protein constitution of the blood (hemolymph) of two species of silk moths, *Hyalophora cecropia* and *Samia cynthia*, during the course of normal development as well as under experimental conditions. The purpose of these studies is to gain insight into the reasons for the changes in protein constitution, as related to development, by examining the proteins with respect to their origin within the organism, the factors influencing their distribution, their possible function, and the role of hormonal factors regulating synthesis.

The blood of silk moths of different developmental stages was analyzed by zone electrophoresis in starch gels. Four major protein components and several minor ones have been resolved from the blood of the *cecropia* pupa in diapause and from blood of the late fifth instar larva of *cynthia*. These bands were followed throughout the life cycle and were found to vary in a precise way with each stage. Protein concentration is maximal in the spinning fifth instar larva of *cynthia*. The number of components drops from four to two in the diapausing *cynthia* pupa, while in diapausing *cecropia* all four bands remain. As the pupa transforms into the adult the number of major bands as well as the total protein concentration drops. Other apparently new and minor components become detectable. Parallel electrophoretic analyses of several organ extracts show that each structure contains a specific complement of protein bands, some of which are similar to those of blood.

Enzymatic activities of the proteins resolved by electrophoresis are demonstrated by histochemical procedures applied according to the methods of Hunter and Markert (1957). A considerable number of enzymatic activities characterize each developmental stage. Esterases with differing substrate and inhibition specificities are found in addition to phosphatases, carbohydrases, sulfatases, tyrosinases, and chymotrypsin. The identification of two esterases as antigens suggests that the other enzymes may also be antigenic. This finding also suggests that the antigens described by Telfer, for which no function is known, may be enzymes.

The histochemical examination of insect blood shows that development, whether in the larva, pupa, or adult, is associated with changes in esterase

activity. One of these esterases can be made to appear in the blood of diapausing *cynthia* pupae by reducing the over-all protein concentration of blood. This particular esterase is presumably synthesized in the gut, since surgical removal of this structure prevents the appearance of the esterase in the blood. Removal of the larval brain lowers the activity of this esterase. The concentration of other proteins is also reduced, suggesting that the concentrations of proteins and enzymes are dependent on hormones.

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MECHANISMS CONTROLLING REPRODUCTION IN TWO VIVIPAROUS COCKROACHES (BLATTARIA)*

Franz Engelmann†
Albert Einstein College of Medicine, New York, N.Y.

It was first demonstrated by Wigglesworth (1936) for *Rhodnius* that egg maturation, that is, yolk deposition in the oöcytes, is dependent on the activity of the corpus allatum. Subsequently it was found that the same principle holds for a variety of species from different insect orders (Weed, 1936; Joly, 1945; Scharrer, 1946; Ozeki, 1949). It soon became apparent that a number of intrinsic and extrinsic factors are involved in the regulation of the function of the corpora allata. For instance, mature or nearly mature eggs in the ovaries seem to reduce the activity of the corpora allata in certain insects (Thomsen, 1940; Day, 1943; Wigglesworth, 1948; Scharrer and von Harnack, 1958). Eggs developing in the brood sac of viviparous cockroaches cause a complete inhibition of the corpora allata (Engelmann, 1957, 1959). Mating in some insects activates the corpora allata and thus stimulates egg maturation (Griffiths and Tauber, 1942; Roth and Willis, 1955, 1956; Engelmann, 1959). In certain other species mating only induces egg deposition and probably does not accelerate egg maturation (Mokia, 1941; Gillett, 1955). Furthermore, food supply is important for the proper functioning of the corpora allata in many insect species, but not in others (Wigglesworth, 1936; Scharrer, 1946; Johansson, 1955; von Harnack, 1958; Larsen and Bodenstein, 1959). Probably humidity, temperature, and light conditions (De Wilde and Stegwee, 1958) are also factors that influence the function of the corpora allata in relation to egg maturation.

We have good evidence that under normal conditions the brain integrates these different afferent stimuli and transforms them into messages to the corpora allata. The brain responds to humoral as well as to nervous afferent stimuli. The integrated information then seems to reach the corpora allata via nervous pathways. The brain apparently regulates not only the activity of the corpora allata but is also the controlling organ for a number of endocrine functions (Scharrer, 1958, 1959).

In the present paper the reproductive patterns of two vivaparous cockroaches, *Leucophaea maderae* and *Diploptera punctata*, will be compared. The elucidation of the control mechanisms for reproduction in these roaches perhaps will facilitate an understanding of the many different features related to reproduction in insects. At the present time no attempt will be made to combine all the known mechanisms into a unifying hypothesis.

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† Present address: Zoological Institute, Johannes Gutenberg Universität, Mainz, West German Federal Republic.

The Function of the Corpora Allata in the Adult Female of Leucophaea and Diploptera

The activation of the ovaries. In adult females of the two viviparous cockroaches, *Leucophaea maderae* and *Diploptera punctata*, periods of egg maturation alternate with long periods of ovarian quiescence. During the latter periods the embryos develop in the brood sac. In both species allatectomy prevents the deposition of yolk in the oöcytes (Scharrer, 1946; Engelmann, 1959). As was shown previously, characteristic histological changes in the corpora allata occur in association with different phases of ovarian activity (Engelmann, 1957, 1959; Scharrer and von Harnack, 1958). When the eggs are maturing, the volume of the corpora allata increases, primarily due to an increase of cytoplasmic volume of its cells; during the following quiescent stage of the ovaries the amount of cytoplasm in the corpora allata is reduced. From these findings it seemed feasible to grade the activity of the corpora allata by calculating the volume of the gland per number of nuclei (Engelmann, 1957). Results are expressed in terms of volume per million nuclei. On the basis of many experiments the following figures represent different degrees of relative activity of the corpora allata. The grouping is of course somewhat arbitrary, but it seems to be quite useful. Values of less than 1.30 mm.^{3} of total gland tissue per million nuclei indicate that this gland is inactive; 1.60 to 2.40 mm.^{3} gland tissue per million nuclei represent an active gland; more than 2.40 mm.^{3} gland tissue per million nuclei indicate highly active corpora allata. FIGURES 1 and 2 demonstrate how the increased cytoplasmic content of the corpora allata parallels the growth of the oöcytes in the ovaries of these species. Basically there is no difference except in the time period needed for egg maturation in the two different species. Details are discussed in previous publications (Engelmann, 1957, 1959).

The activation of the accessory sex glands. It was previously shown that allatectomy prevents the activation of the accessory sex glands (Scharrer, 1946) that normally produce the materials used in the formation of the cockroach oötheca. These glands function independently from the ovaries, since castration does not prevent their activity.

Of 25 females of *Leucophaea* in which the accessory sex glands located anteriorly on the bursa copulatrix (A1) were removed (Engelmann, 1957*b*), one extruded the spermatophore on the twelfth day and two on the thirteenth day after mating; the other animals kept the spermatophore until the expected time of ovulation. These females could not ovulate because the spermatophore blocked the passage of the eggs. In 16 unoperated females the spermatophore was extruded between 7 and 12 days after mating. All of 20 sham-operated animals extruded the spermatophore by the fourteenth day after copulation; the operation merely caused a slight delay of the extrusion. Seven females from which the gland A1 was removed unilaterally also extruded the spermatophore somewhat later than unoperated females, but by the fourteenth day after mating all except one had done so; the last extruded it on the seventeenth day. In eight females, the removal of the large glands (A2 and A3) had no effect on the time of extrusion of the spermatophore. Thus it

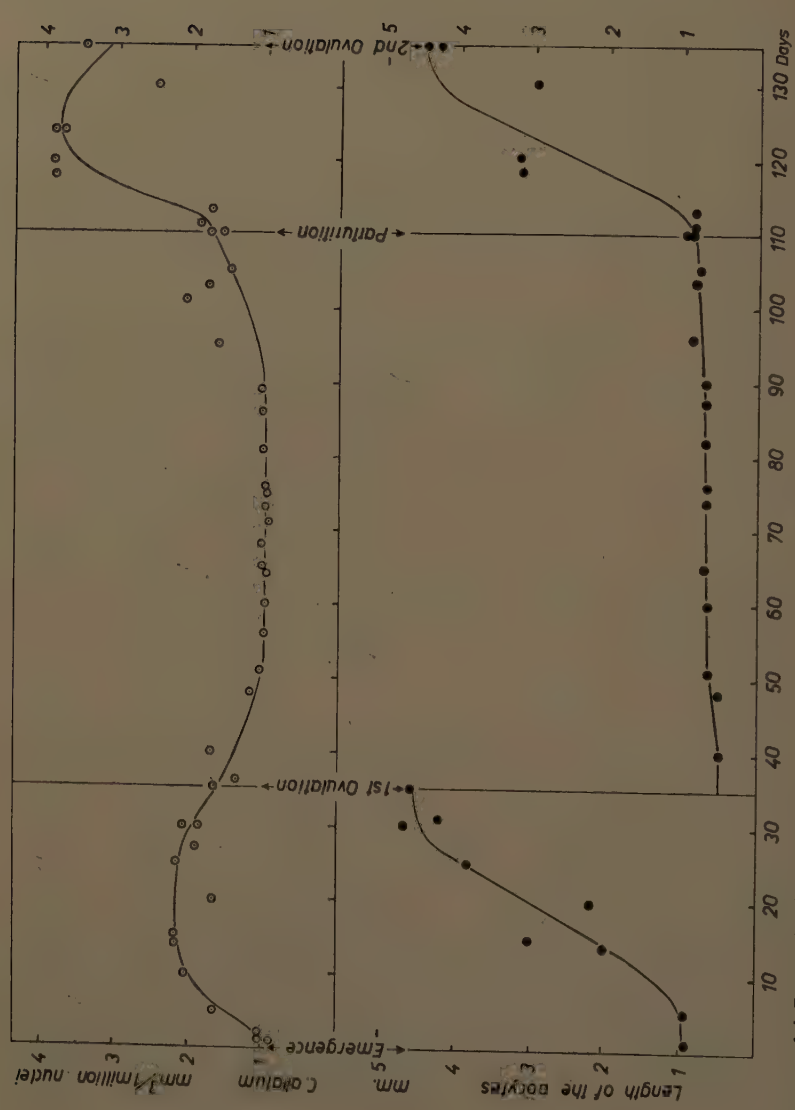

FIGURE 1. Diagram indicating the morphological changes in the corpora allata of *Leucophaea* (*upper curve*), which are correlated with the growth of the terminal oöcytes of the ovarioles (*lower curve*) during the first preoviposition period, pregnancy, and second preoviposition period.

FIGURE 2. Diagram indicating the morphological changes signalling cyclic activity in the corpora allata of *Diploptera* (*upper curves*), which are correlated with the growth of the oöcytes (*lower curves*) during the first and second preoviposition periods. Reproduced by permission from *Biological Bulletin* (Engelmann, 1959).

seems that the secretion of the A1 accessory sex glands facilitates the extrusion of the spermatophore 7 to 12 days after mating. The secretory product of this same gland probably also contributes to the materials from which the egg case is built. Since the accessory sex glands are under the control of the corpora allata no extrusion of the spermatophore occurred in 15 allatectomized females tested in this connection.

That spermatophore extrusion is facilitated by the secretion of the anterior pair of accessory sex glands can also be demonstrated in *Diploptera*. In this species unoperated females extrude the spermatophore by the sixth day after the first mating. This pair of glands was removed from six females of *Diploptera* shortly after mating. None of these females extruded the spermatophore within the test period of two weeks. The remaining spermatophore blocked the ovulation of the mature eggs by preventing the eggs from passing the bursa copulatrix.

The control of mating behavior. An incidental observation led to the finding that the response of the female of *Leucophaea* to the courting male is largely under the control of the corpora allata. Allatectomy in females shortly after emergence caused the animals to respond to the courting males less readily than normal females. Only about 30 per cent of 36 operated females mated within the period of 26 days after emergence, whereas 90 per cent of control animals mated in the same interval (Engelmann, 1960). The reimplantation of active corpora allata in allatectomized females restored their normal responsiveness to courting males; 82 per cent of these females mated within 12 days (11 animals) after reimplantation. Of 14 allatectomized animals that received sham implants, only 33 per cent mated within the given time interval. Clearly, active corpora allata increase the readiness with which the female accepts the male. Ovariectomy had no effect on mating behavior.

It is obvious from the given data that allatectomy does not completely eliminate mating in the female; it merely reduces the percentage of females that will mate during a given period of time. It seemed likely, therefore, that the corpus allatum hormone may act by lowering the threshold for the perception of the male odor, because only after the female has perceived this odor will she accept the male (Engelmann, 1960).

During this study of mating behavior in *Leucophaea* it became apparent that mating in 80 females took place when the largest oöcytes in the ovaries had not yet surpassed an average length of 1.08 $\pm$ 0.01 mm. The full length of the mature oöcyte is 4.5 to 5.0 mm. This finding indicated that the corpora allata at the time of mating are probably only moderately active; this was actually found in six cases selected at random (index of activity: 1.31 $\pm$ 0.02 mm.^{3} gland tissue per million nuclei).

Additional but indirect support for this conclusion was obtained by observing the length of time between mating and ovulation in 70 females. This was an interval of 21.1 $\pm$ 0.03 days. Thus the time needed for the completion of egg maturation was fairly constant whether the first mating took place 10 or 40 days after emergence (FIGURE 3). This period, however, is constant only if the eggs in the ovaries are still small at the time of mating. It applies only if the females had constant access to males during the entire period. The regression

coefficient for this correlation has the value $b = 0.9651$. If the interval between mating and ovulation were constant, the theoretical regression coefficient should be $b = 1$. A statistical analysis of the data revealed a slight but not very significant shortening of the interval when the females mated later after emergence ($P < 0.05$). This slight shortening of the period needed for egg maturation might not be due to the presence of larger oöcytes at the later mating; it could

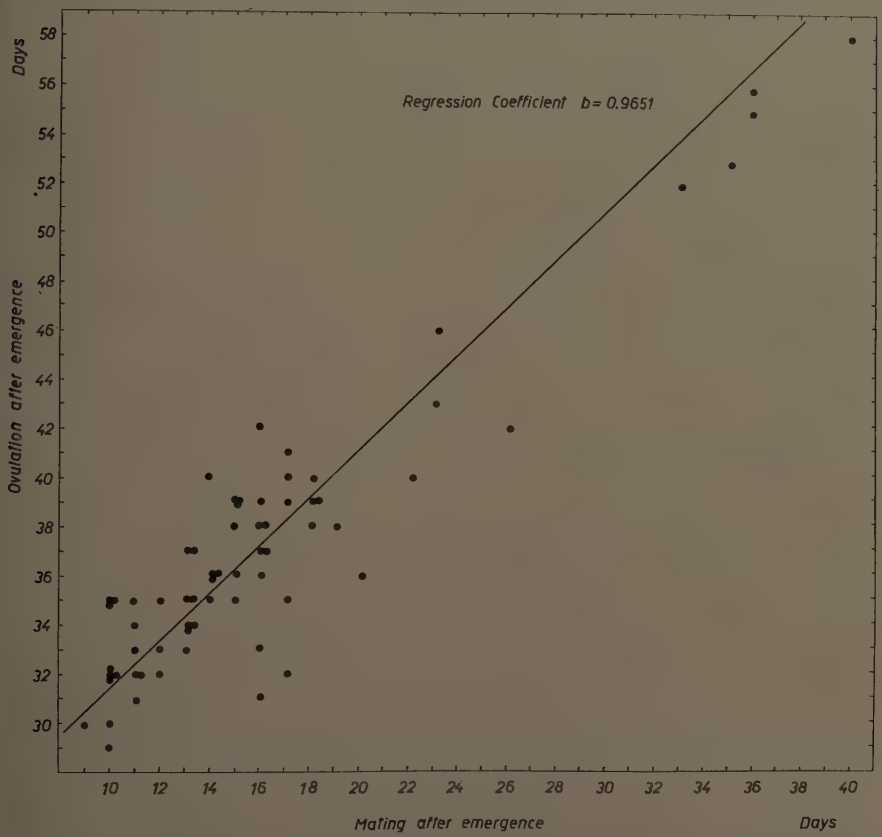

FIGURE 3. Correlation between time of mating and ovulation after emergence in *Leucophaea*. The regression coefficient was calculated from data obtained in 70 animals.

be explained by the presence of larger amounts of reserve substances that might allow a faster growth of the eggs.

One point that seems to emerge from these observations should be stressed. The corpus allatum hormone must be present in low titer to stimulate the responsiveness of the female. A high titer of the hormone has no effect since, from a total of 80 females, none mated that had oöcytes exceeding a size of 1.46 mm. Apparently as soon as a certain titer is surpassed, the female does not accept the male any more. It was occasionally observed that females did not mate even with ready access to males. Those females often matured their eggs within the range of mated females. The reasons why some females did not mate at the "right time" are unknown.

In *Diploptera* a participation of the corpora allata in the mating behavior of the females seems highly improbable, since the female is rather inactive during mating (Roth and Willis, 1955). Allatectomy had no effect on mating in eight females of this species. Operated females mated as readily as intact animals.

The Control of the Activity of the Corpora Allata by the Brain in Leucophaea and Diploptera

In *Leucophaea* and *Diploptera* the corpora allata are innervated by the brain in the same way as in many other insect species (Cazal, 1948). Severance of the nervi allati in animals that have inactive corpora allata, for example, in pregnant females of *Leucophaea* or in virgins of *Diploptera*, results in activation of the corpora allata. Maturation of the eggs follows (Scharrer, 1952; Engelmann and Lüscher, 1956; Engelmann, 1959). These experiments demonstrate that the corpora allata in these species are inactivated by nervous impulses during certain periods of the reproductive cycle.

It was previously reported (Engelmann, 1957) that isolated corpora allata in *Leucophaea* did not induce egg maturation within the same interval as did severance of the nervi allati alone. This observation needed further exploration. It was interesting to know whether inactive corpora allata can become active and induce egg maturation after a long time interval, and whether isolated active corpora allata may remain active for several months and cause two or more cycles of egg maturation. To test these possibilities in a large number of pregnant females isolated active or inactive corpora allata taken from last instar nymphs at different time intervals after molt were implanted. These "isolated" corpora allata had a piece of corpora cardiaca attached in order to handle the implants better; they were completely isolated from the central nervous system. The hosts were observed for several months, some of them up to eight months.

Of 60 pregnant females that received four active corpora allata each, 54 animals exhibited complete maturation of the first batch of eggs within 46 days after implantation; five additional animals matured the eggs by the sixtieth day. Since the corpora allata of these pregnant females are inhibited, this result shows that in 98 per cent of the cases egg maturation was induced by the implants. On the other hand, of 70 pregnant females that received four inactive corpora allata each, only 27 animals matured eggs within 46 days and 17 additional animals by the sixtieth day. Thus only 63 per cent of these implants induced egg maturation. The results of this experiment indicate that many of the isolated inactive corpora allata do eventually become active and elicit yolk deposition. However, complete maturation of the eggs takes on the average somewhat longer than after implantation of active corpora allata. The reason why nearly 40 per cent of the inactive isolated corpora allata did not become active after two months is not known.

Of further interest was how isolated active or inactive corpora allata behave over a period of eight months. Samples were taken from both series at intervals. The degree of activity of the implanted corpora allata as well as of the host corpora allata was checked histologically, and a correlation with the degree of egg maturation was looked for. The samples were grouped according to the

stage of oöcyte maturation. The following interesting picture emerged. After implantation of active corpora allata (average activity of a sample of 12 corpora allata: 1.86 ± 0.06 mm.^{3} gland tissue per million nuclei) an early maturation of the eggs could be detected six to eight days later. Between 16 and 25 days, when the rate of yolk deposition was highest, the implants were very active when judged by histological criteria. As soon as the eggs were mature (4.5 to 5.0 mm.) the old egg case still in the brood sac was prematurely extruded. The embryos were not yet ready to hatch. Two or three days later the eggs were ovulated and the newly formed egg case was retracted in the brood sac. The implanted corpora allata in 14 animals checked at the time of extrusion of the old egg case apparently had dropped in activity, as judged by the decrease of their cytoplasmic volume (average 1.69 ± 0.04 mm.^{3} gland tissue per million nuclei, in 49 recovered glands). During the following induced pregnancy the implants dropped further to complete inactivity (average 1.17 ± 0.04 mm.^{3} gland tissue per million nuclei, in 17 recovered corpora allata in five animals). However, before the embryos in the brood sac were ready to hatch, the next set of eggs matured. The implanted corpora allata must have become active once more. Again, at the time of extrusion of the egg case from the brood sac, the implanted corpora allata were decreasing in their activity. In 21 implants recovered from six females, the activity ratio of 1.51 ± 0.06 mm.^{3} gland tissue per million nuclei was calculated. Glands of such a degree of activity would not induce complete egg growth as we know from other experiments. These glands must have had higher activity in the immediately preceding period.

These findings indicate that corpora allata isolated from the central nervous system show a somewhat cyclic activity independent of any nervous control. This apparent cyclic activity in some animals was repeated up to five times within eight months. Usually isolated corpora allata still had a perfectly healthy appearance after many months.

Isolated inactive corpora allata were observed in a similar fashion. Those implants that became active once (as judged by induced egg maturation), behaved basically in the same way as isolated active corpora allata. They went through a histologically detectable cyclic activity and induced periodic egg growth.

It is apparent that the cyclic activity of isolated corpora allata in *Leucophaea* does not permit normal development of the embryos. The embryos are always extruded from the brood sac prematurely. In the intact animal, therefore, the innervation of the corpora allata assures an activity of the corpora allata at the "right time."

Nothing is known about the behavior of isolated corpora allata in *Diploptera*. It was previously reported (Engelmann, 1959), however, that after severance of the nervi allati in this species a rapid maturation of two batches of eggs can occur in succession.

Stimuli Activating the Corpora Allata

Mating in Leucophaea. Mating normally takes place between the 11th and 19th day after emergence; none of 184 observed females mated before the eighth

day, seven females mated after the 26th day. Ovulation in mated females was recorded 36.1 ± 0.5 days after emergence (70 randomly selected females). On the other hand, ovulation in 66 virgins occurred 69.3 ± 13.2 days after emergence. Thus there was a significant delay in egg maturation if mating was prevented, since there is no evidence that mature eggs remain in the ovaries of virgins. Furthermore, virgins exhibit great variability in the period needed for egg maturation. Of the 66 virgins, 18 ovulated within the range of mated animals, whereas some others did so as late as five months after emergence. Six additional females had not ovulated when the observation was discontinued 123 to 250 days after emergence. Thus mating shortens the period needed for egg maturation, so that ovulation takes place at a rather definite period after emergence. The afferent stimuli of mating apparently enhance the activity of the corpora allata.

It was furthermore of interest to know whether, after removal of the egg case from pregnant females, mating is also essential for a normal rate of egg growth. In 30 pregnant females, the egg cases were removed 29 to 34 days after the

Table 1

Effect of Mating After Egg Case Removal in Females of *Leucophaea*

	Number of animals	Next ovulation (days after operation)*	Significance of acceleration of egg maturation (t test)
Removal of egg case 0–1 day after ovulation.	18	64.7 ± 1.9	—
Removal of egg case on day of ovulation; followed by mating.	18	56.2 ± 0.9	$P < 0.01$
Removal of egg case 29–34 days after ovulation.	16	35.6 ± 1.0	—
Removal of egg case 29–34 days after ovulation; followed by mating.	14	34.9 ± 0.9	—

* Numbers following ± here, and in other tables with this paper, are standard errors.

previous ovulation. Of these females 14 were allowed to mate and the remaining 16 females were kept isolated. In both series ovulation was recorded about five weeks later (TABLE 1). This finding agrees with previous observations that after parturition mating does not accelerate egg maturation (in 46 animals the next ovulation occurred 22.1 ± 0.9 days later). On the other hand, if the egg cases were removed immediately after ovulation (36 animals) and this was followed by mating (18 animals), then a significant shortening ($P < 0.01$) of the period of egg maturation resulted (TABLE 1). These findings lead me to conclude that in *Leucophaea* mating is not necessary to induce egg maturation at a normal rate after parturition or after egg case removal. However, the situation that is observed if the egg cases were removed shortly after ovulation might indicate that there are additional unknown factors involved, which are operating only for a short time after ovulation.

The fact that mating stimulates the activity of the corpora allata suggests that these stimuli are received in the genital apparatus and ascend via the ventral nerve cord to the brain. This assumption may be tested if one severs the ventral nerve cord shortly after mating. The severance of the ventral nerve cord in 19 females 0 to 48 hours after mating resulted in a significant delay of

egg maturation compared with the situation in intact females ($P < 0.01$). Ovulation in these animals was recorded 29.8 ± 0.9 days after mating, or 43.3 ± 1.5 days after emergence. In 70 normal females ovulation took place 21.0 ± 0.3 days after mating, or 36.1 ± 0.5 days after emergence. The severance of the ventral nerve cord more than two days after mating in 29 females had no delaying effect on egg maturation (TABLE 2). This experiment clearly suggests that mating stimuli are received in the genital apparatus of the female and that these stimuli are transmitted to the brain via the ventral nerve cord, and then to the corpora allata. The same experiment also indicates that the mating stimuli do not consist of a transient stimulation, since the full mating effect is observed only if the ventral nerve cord of the female remained intact for at least two days after mating.

In another experimental series, the ventral nerve cord was severed in seven virgins 14 days after emergence, about the time when the average female will mate. These females, which were not allowed to mate after operation, ovulated 44.8 ± 1.7 days after emergence. The time needed for egg maturation in

TABLE 2

EFFECT OF SEVERANCE OF VENTRAL NERVE CORD IN VIRGIN OR MATED FEMALES OF *LEUCOPHAEA*

	Number of animals	Ovulation (days after emergence)*
Control: virgins.	66	69.3 ± 13.2
Severance of nerve cord in virgins 14 days after emergence.	7	44.8 ± 1.7
Severance of nerve cord 0–2 days after mating.	19	43.3 ± 1.5
Severance of nerve cord 3–19 days after mating.	29	34.1 ± 0.8
Control: mated females.	70	36.1 ± 0.5

* See footnote for TABLE 1.

this series was roughly the same as after mating and immediate severance of the ventral nerve cord. From this I must conclude that the severance of the ventral nerve cord either stimulates the corpora allata or cuts off an inhibitory center for the corpora allata. The latter possibility seems to be the more likely. This hypothetical inhibitory organ for the corpora allata would have to be located posterior to the level of cord severance. The result in the series where mating was followed by cord severance, reported in the previous paragraph, can now be interpreted as the effect of nerve severance alone. It should be emphasized, however, that the effect of mating on the corpora allata is stronger than the effect of cord severance since, with the latter, egg maturation takes significantly longer than after mating ($P < 0.01$). This indicates that the actual mechanism of the perception of mating stimuli is probably more complex than we appreciate at the present time.

One might furthermore assume that the gonapophyses, which are supplied with numerous sensory sensillae, are the primary site of perception of the mating stimuli. To test this point, the three pairs of gonapophyses of 16 virgins were completely excised 2 to 11 days after emergence. No mating took place in these animals during the period of observation. However, in 39.5 ± 1.5

days after emergence, the time interval almost normal for mated females, 15 of these virgins developed mature eggs; one animal had not deposited any yolk in the oöcytes within that time. It seems that the afferent nerves originating from the sensory receptors located on the gonapophyses were briefly stimulated by excision of the gonapophyses. This stimulation had a rather long-lasting effect on the corpora allata, which in turn caused egg maturation within a normal time interval. The question whether females without gonapophyses would still perceive mating stimuli if mating would occur is pointless, since mating could not accelerate egg maturation further than it normally does; therefore, we would have no means to detect the mating effect in this case.

The effect of excision of the gonapophyses on the corpora allata suggests that the mating stimuli in *Leucophaea* are of mechanical nature. Females that were mated with castrated males (33 animals) matured their eggs within 21.6 ± 0.4 days, or 35.9 ± 0.9 days after emergence. Since this is the normal time, apparently no humoral factor originating from the sperm mass is involved in the transmission of the mating "information" to the brain.

Food intake in Leucophaea. Food intake is known as another factor that stimulates egg maturation in *Leucophaea* (Scharrer, 1946; Johansson, 1955). It seemed probable that the nutrient value of the food stimulates the activity of the corpora allata. However recent experiments reported by Larsen and Bodenstein (1959) suggest the possibility that in certain species mechanical distention of the gut may serve as the stimulus. To test this point, virgins of *Leucophaea* were subjected to various treatments. As noted above, virgins mature their eggs more slowly than mated females and, therefore, any alteration in the speed of this process after experimental treatment is readily detected.

A mechanical distention of the mid-gut was caused in 15 virgins either by ligation of the hind-gut or by sealing the anus shortly after emergence. These animals were observed for 27 days. After a few days they all had tremendously distended mid-guts, since they continually ate some food. It was noticeable, however, that these animals ate less than normal virgins. At the termination of the experiment the largest oöcytes in the ovaries measured on the average 1.15 ± 0.14 mm. Oöcytes in 13 virgins at the same age measured 1.99 ± 0.25 mm. Thus, in spite of an enormous distention of the mid-gut egg maturation was not accelerated, but slowed down.

A distention of the abdomen in seven virgins was artificially caused by placing large paraffin pellets into the body cavity. In these animals also egg maturation did not surpass the normal level of the 26th day after emergence. The average length of the oöcytes in these experimental animals was 1.00 ± 0.02 mm. Thus, there is no evidence that, in *Leucophaea*, distention of either mid-gut or abdomen activates the corpora allata. The effect of food intake on the corpora allata is therefore probably due to the nutrient value of the food. Since animals of the three experimental series ate less than normal ones, the slower egg maturation probably reflected a reduced activation of the corpora allata.

The data given above suggest that starved females of *Leucophaea* do not mature their eggs because the corpora allata are practically inactive (see also von Harnack, 1958). On the other hand mating stimulates the activity of the corpora allata. It was therefore of interest to see how females that are sub-

jected to both treatments might react. Nineteen starved females were exposed to normal males. The males were exchanged every second or third day to assure that the courting behavior of the males was not impaired by starvation. Seven of the 19 females mated within 26 days after emergence. The length of the oöcytes in all females was measured at the termination of this test period. It was found that neither the mated nor the virgin females of this series had begun to deposit yolk in the oöcytes; the average length of the oöcytes was 1.01 ± 0.02 mm. From this it is obvious that the brain properly integrated these different afferent stimuli into messages to the corpora allata; no activation of the corpora allata resulted.

Mating and parturition in Diploptera. As previously reported, mating is essential for the deposition of yolk in the oöcytes at a normal rate in *Diploptera* (Roth and Willis, 1955; Engelmann, 1959). Mating in this species normally occurs immediately after emergence and is followed by ovulation of mature eggs about 10 days later. Among 45 virgins, on the other hand, the earliest ovulation was observed 37 days after emergence. Many of these females had not ovulated several months later (Engelmann, 1959). Thirty-five virgins have now been observed for their entire lifetime, and 14 of these females had not ovulated even when they died. The average life span of those females that never matured their eggs was 166 days and thus much shorter than the average life span of 307 days of females that developed mature eggs at least once. Perhaps some animals of the first group would have matured their eggs had they lived longer. Nevertheless it may be concluded that if mating in this species does not occur there may be a complete absence of egg maturation throughout the lifetime of the animal. Mating therefore is an important factor that stimulates the activity of the corpora allata. As in *Leucophaea*, it is the mechanical stimulus of mating that activates the corpora allata (Engelmann, 1959). The mating stimuli are transmitted to the brain and corpora allata via the ventral nerve cord, since a severance of the cord before mating results in a complete absence of egg maturation; in females that did not mate after cord severance egg maturation failed completely.

It was also previously reported that parturition, like mating, apparently induces growth of the eggs at a normal rate in *Diploptera* (Engelmann, 1959). In 14 pregnant females, the egg cases were removed on the second or fifth day after ovulation. If no mating then occurred, the next ovulation was recorded between 42 and 96 days later. A similar result was obtained in five additional pregnant females from which the egg cases were removed on the 30th day after ovulation. However if mating followed shortly after the removal of the egg case, the next batch of eggs matured within 10 days (five animals). This indicates that without a mechanical stimulation of the genital apparatus eggs do not mature at a normal rate. The removal of the egg case from the brood sac is not a stimulus that is comparable to mating. Such females behave like virgins. Since, on the other hand, in 47 females the next ovulation was recorded 6.1 ± 0.3 days after parturition it is obvious that parturition serves as a stimulus. Further proof was sought for this conclusion by removing the egg cases from pregnant females (16 animals) 2 to 10 days before parturition would have been expected. It is, of course, impossible to determine exactly when parturition would have taken place. Stimulation of the genital apparatus was avoided

during the removal of the egg cases. Five of the experimental animals ovulated within the period normally needed for egg maturation (10 days); two additional females did so two weeks after the removal of the egg cases and five animals after three weeks. Four animals had not matured their eggs when the experiment was discontinued five weeks after the removal of the egg cases; this is the time at which these animals surely would have matured eggs if parturition had taken place. Probably maturation in the successful cases was already initiated at the time of the removal of the egg cases, so that it had only to be completed (Engelmann, 1959). This suggests that the stimulation of the genital apparatus prior to parturition by the movements of the egg case may suffice to induce complete egg maturation. This experiment also confirms previous observations that parturition as such is a stimulus to the corpora allata, since some females did not respond immediately after gentle removal of the egg case. When the egg case is removed early enough from pregnant females these animals behave like virgins.

In *Diploptera* nothing has been known about the food requirements for the maturation of eggs. In this species eggs mature only if the females have mated. Therefore, 10 females that had copulated immediately after emergence were taken from the stock colony and kept without food. All of these females matured their eggs and most ovulated between 11 and 15 days after emergence. Thus, in *Diploptera* food intake is not necessary to guarantee complete maturation, at least of the first batch of eggs. There was only a slight delay for complete egg maturation. Mating alone stimulates the corpora allata sufficiently. But it should be kept in mind that in this situation, in contrast to my experiment in *Leucophaea*, reported above, mating occurred before the animals were subjected to inanition, and therefore only one factor was acting on the corpora allata at a time.

Stimuli Inhibiting the Function of the Corpora Allata

The presence of the egg case in the brood sac of Leucophaea. The corpora allata and, hence, egg maturation, are inhibited during development of the embryos, which lasts for about two and a half months (Engelmann, 1957). There are two principal mechanisms that might account for the inactivation of the corpora allata during pregnancy. Perhaps receptors in the genital apparatus are mechanically stimulated by the egg case, and this inhibitory information is transmitted to the brain via the ventral nerve cord. On the other hand, a humoral agent released by the egg case might, either alone or in addition to the mechanical factor, act on the brain and in turn on the corpora allata. Experimental evidence favored the latter possibility (Engelmann, 1957). However, in view of the more recent experiments of Roth and Stay (1959) the question was taken up again.

To test the first of the two possible mechanisms involved, the ventral nerve cord was severed 31 to 37 days after ovulation in 19 pregnant females. In contrast to the previously reported data (Engelmann, 1957), these animals later began to mature the next batch of eggs and ovulated 39.1 $\pm$ 1.4 days after operation. The old egg case was always extruded on the average 2 to 3 days before ovulation took place. The removal of the egg cases from 30 preg-

nant females 29 to 34 days after the previous ovulation resulted in the maturation of the next batch of eggs at 35.2 ± 0.7 days (TABLE 3). These data seemed to support the idea that the presence of an egg case in the brood sac was somehow transmitted to the brain via the ventral nerve cord. A statistical treatment of the data revealed a significant delay of egg maturation ($P < 0.01$) after the severance of the ventral nerve cord, compared with the timing in animals from which the egg cases were removed. This point was further supported by an extension of the present experiment: seven of the 19 females whose ventral nerve cord had been severed were observed until a second batch of eggs had matured after operation. Those females may be considered here as operated on the day of ovulation. The mature eggs ovulated 73.4 ± 1.5 days after the previous ovulation. The embryos were prematurely extruded two to three days before the next eggs ovulated. Three additional operated females that had extruded the first egg case right after the first ovulation following operation were also observed until the next eggs had matured. These latter females are comparable to animals from which the egg cases were removed on the day of ovulation (TABLE 3). These females ovulated by the 63rd day after the

TABLE 3

EFFECT OF SEVERANCE OF VENTRAL NERVE CORD IN PREGNANT FEMALES OF *LEUCOPHAEA*

	Number of animals	Operation (days after ovulation)	Next ovulation (days after operation)*	Significance of delay in egg maturation (*t* test)
Control: removal of egg case.	30	29–34	35.2 ± 0.7	—
Severance of nerve cord.	19	31–37	39.1 ± 1.4	$P < 0.01$
Control: removal of egg case.	18	0–1	64.7 ± 1.9	—
Severance of nerve cord.	7	0	73.4 ± 1.5	$P < 0.02$

* See footnote for TABLE 1.

previous ovulation. Thus we find that egg maturation took significantly longer in females whose ventral nerve cord was severed at the time of ovulation as compared with females whose egg cases were removed at ovulation ($P < 0.02$). This last experimental series indicates also that a severance of the ventral nerve cord in pregnant females has an effect on the corpora allata that lasts through two periods of egg maturation at least. Perhaps, then, the inhibitory center of the corpora allata was cut off in this experiment. In conclusion it may be postulated that mechanical stimulation of the genital apparatus, different from the stimulation at mating, accounts primarily for the inhibition of the corpora allata during pregnancy. This stimulation is transmitted to the brain via the ventral nerve cord. It would appear, however, that additional factors play an important role in the maintenance of pregnancy.

Of what nature are these additional factors? One factor to be considered was that an agent may be given off by the egg case in the brood sac during embryonic development, since the implantation of an egg case in the body cavity of newly activated females prevented egg maturation for some time (Engelmann, 1957). To test this point once again, egg cases of no more than 20 days of age were homogenized in Ringer's fluid. The homogenate was centrifuged for 10

minutes and then 0.1 ml. of the clear supernatant was injected into females whose egg cases were removed at the time (TABLE 4). Every fifth day for 30 days 0.1 ml. extract was injected. In the 19 females treated in this manner, the oöcytes at 30 days were only 1.70 ± 0.19 mm. long, whereas in controls the eggs had a length of 4.70 ± 0.15 mm. Thus an inhibition of the corpora allata can be demonstrated by the application of egg case extract.

It was of interest to learn more about the nature of this inhibitory substance in homogenates of egg cases. In a control experiment seven females were treated in a way similar to that described above, but these females received six injections of 0.1 ml. of muscle homogenate. Surprisingly enough, in these females the corpora allata were inhibited almost to the same extent as when egg case extract was given (TABLE 4). From this it might be concluded that perhaps a nonspecific substance causes an inhibition of the corpora allata during pregnancy. Possibly the same explanation is true for the observation that eggs in resorption inhibit the corpora allata (Engelmann, 1957).

It was of further interest to see how females behaved after exposure to two

TABLE 4

EFFECT OF INJECTION OF EXTRACTS AFTER REMOVAL OF EGG CASE 28 TO 36 DAYS AFTER OVULATION IN *LEUCOPHAEA*

	Number of animals	Animals checked (days after operation)	Ovary (length of the largest oöcytes, in mm.)*
Control: pregnant females.	13	—	0.69 ± 0.02
Injection of 0.1 ml. of egg case extract every 5th day.	19	30	1.70 ± 0.19
Injection of 0.1 ml. of muscle homogenate every 5th day.	18	30	2.12 ± 0.20
Control: removal of egg case.	16	30	4.70 ± 0.15

* See footnote for TABLE 1.

stimuli of opposite quality at the same time. In eight females the gonapophyses were excised 30 to 36 days after ovulation. The excision of the gonapophyses in virgins is known to stimulate the corpora allata. Three of these females extruded the egg case two to four days later; however, five females kept the egg case. The latter were checked 30 days after operation. None of them had deposited any yolk in the oöcytes. Clearly, the excision of the gonapophyses did not stimulate the corpora allata. The brain apparently integrated the different stimuli properly. The stimuli exerted by the egg case in the brood sac dominated.

The presence of the egg case in the brood sac of Diploptera. Mechanical and humoral factors apparently regulate the activity of the corpora allata in *Leucophaea* during the period of pregnancy. What do we know about these mechanisms in *Diploptera*? As previously shown, removal of the egg case in pregnant females of *Diploptera* does not result in an immediate activation of the corpora allata, as it does in *Leucophaea*. The corpora allata in *Diploptera* are apparently inhibited through some mechanism that does not originate primarily in the egg case present in the brood sac. On the other hand, the corpora allata

do not stay inactive after egg case removal as long as they would under normal conditions, that is, until the expected time of parturition; the next batch of eggs matures earlier. It was therefore concluded that the egg case in the brood sac has some influence on the corpora allata that maintains their inactivity throughout the whole period of pregnancy.

In analogy with what we know in *Leucophaea* the nature of the influence of the egg case on the corpora allata could either be mechanical or humoral or both. To eliminate one of these factors, the ventral nerve cord was severed at different intervals after ovulation: in 54 of these females the ventral nerve cord was successfully severed two days after ovulation. All of these females extruded the egg case by the 45th day after operation or died; most females extruded the egg case before the 30th day. Every animal was dissected on the day of extrusion of the egg case and it was found that none had even begun to deposit yolk in the oöcytes. The corpora allata apparently did not become active within that time interval. The reasons why these females extruded the egg case are unknown. Nine females that were unsuccessfully operated completed their pregnancy and gave birth to young. The next egg maturation was initiated in these animals at the same time as in normal females.

The severance of the ventral nerve cord in pregnant females had no effect on the corpora allata. However no effect could be expected within the test period, because removal of the egg case did not result in any response within that same period either. In an additional experiment the ventral nerve cord of eight pregnant females was severed 30 to 40 days after ovulation. Again the corpora allata did not respond and, consequently, no egg maturation took place within the next 8 to 24 days (the oöcytes measured 0.06 ± 0.01 mm.). Of 14 pregnant females, however, that were operated on 50 to 75 days after ovulation, 12 animals exhibited an effect of the cord severance on the corpora allata. These 12 females began to mature or completely matured their eggs within 9 to 27 days. Certainly some of these females had initiated egg maturation before the experiment was performed and thus only completed it. In all probability, however, some of these animals were still too young and, therefore, maturation of eggs must have been initiated by the severance of the ventral nerve cord. It is obscure why only in this last series did severance of the ventral nerve cord cause an activation of the corpora allata in most of the animals. Earlier the females would have been capable of being activated, as was shown if mating was allowed after egg case removal (Engelmann, 1959). Perhaps during late pregnancy the female is more sensitive and, therefore, can be activated more readily. Be that as it may, it may well be concluded that some nervous component is involved in the maintenance of pregnancy, but perhaps only during the last phase of pregnancy. Whether humoral factors are also involved is unknown.

Discussion and Conclusion

The reproductive cycles and their control in the two viviparous cockroaches discussed in this paper follow basically the same principle. However, there are a variety of differences, sometimes perhaps only in degree of effectiveness. The following sequence of events is observed. Mildly active corpora allata stimulate the female of *Leucophaea* to accept the courting male. In *Diploptera*

mating takes place without the intervention of the corpora allata. In both species, the mating stimuli received by sensory receptors in the genital apparatus ascend via the ventral nerve cord to the brain. These impulses counteract the usual inhibitory influence of the brain on the corpora allata. In *Diploptera* the role of the mating stimuli is more pronounced than in *Leucophaea*. The corpora allata then produce and release gonadotrophic hormone, which induces the maturation of the eggs and also activation of the accessory sex glands. After ovulation the mature eggs are deposited in the brood sac where the entire embryonic development takes place. During this pregnancy the corpora allata are completely inhibited via the nervi corporis allati. The presence of the eggs in the brood sac, therefore, must influence the brain, which in turn acts on the corpora allata. The inhibitory principle is not definitely known; it may be either humoral or mechanical or even both. After parturition eggs mature without preceding copulation. In *Diploptera* the hatching young stimulate the genital apparatus sufficiently. In *Leucophaea* the extrusion of the egg case at parturition suffices to induce activation of the corpora allata.

What features of the reproductive cycles in these viviparous roaches can be found also in other insects? Egg maturation is known to be dependent on the activity of the corpora allata in a number of species of different orders. This was shown by allatectomy and reimplantation experiments in Orthoptera (Weed, 1936; Pfeiffer, 1939; Scharrer, 1946), Dermaptera (Ozeki, 1949), Hemiptera (Wigglesworth, 1936; Johansson, 1954, 1958), Coleoptera (Joly, 1945, 1950; De Wilde and Stegwee, 1958), and Diptera (Thomsen, 1940, 1942; Day, 1943; Detinova, 1945; Bodenstein, 1947; Gillett, 1956; Larsen, 1958). In many publications a structural change in the corpora allata during periods of deposition of yolk in the oöcytes has been reported.

In *Leucophaea* and *Diploptera* the brain restrains the activity of the corpora allata by way of the nervi corporis allati during certain periods of the reproductive cycles. Severance of these nerves always results in an activation of the corpora allata (Scharrer, 1952; Engelmann and Lüscher, 1956; Engelmann, 1959). Apparently in other insect species a similar nervous inhibition exists. For instance, in *Oncopeltus* the corpus allatum is inhibited by the brain during starvation (Johansson, 1958), and severance of the nervi allati causes activation of the corpus allatum and egg maturation. In flies severance of the nerves to the ring gland results in a slight hypertrophy of the corpus allatum (Day, 1943).

In all the cases mentioned above the response of the corpora allata was studied only for a brief period after operation. One may ask how corpora allata, totally isolated from the central nervous system, behave in the course of several months. In *Leucophaea* isolated corpora allata, which had only a little piece of corpora cardiaca attached, were observed over a period of eight months and it was shown that these glands undergo a cyclic activity. After each peak of activity the corpora allata drop to inactivity without any nervous restraining influence. A few weeks later these corpora allata are again active, as shown by histological criteria and by the maturation of eggs in the ovaries. This cyclic activity of the corpora allata is not correlated with the developmental stages of the embryos in the brood sac. The latter cannot complete their development, because they are extruded from the brood sac prematurely. Thus

it appears that normally the "intrinsic cyclic activity" of the corpora allata is kept in check by nerves from the brain, and thereby coordinated with the reproductive phases of the animal. These findings clearly demonstrate the importance of proper innervation of the corpora allata in *Leucophaea*. The same principle may hold for other species. Transplanted active corpora allata of *Rhodnius* induce only one or two extranymphal molts (Wigglesworth, 1948). In *Dixippus* the corpora allata likewise seem to become inactive after long periods of isolation (Pflugfelder, 1939, 1940). In these cases isolated corpora allata apparently do not maintain their activity. Whether in *Rhodnius* or *Dixippus* the corpora allata would have become active again after still longer periods is not known.

As the experiments in *Leucophaea* indicate, the brain is the final regulator of the function of the corpora allata throughout the reproductive phases. The brain is the integrating organ for the afferent informations, since stimuli shown to act under normal physiological conditions on the corpora allata reach the brain first. The same condition applies in *Diploptera*. There, for example, stimuli received in the genital apparatus during mating or parturition reach the brain via the ventral nerve cord and induce a cessation of the inhibition of the corpora allata (Engelmann, 1959). Severance of the ventral nerve cord prior to mating in this species completely prevents egg maturation. In *Leucophaea*, where the stimulation received during mating is important for egg maturation, although less pronounced, these stimuli likewise ascend via the ventral nerve cord to the brain.

In *Leucophaea* it was noticed that mating stimuli do not consist of a transient stimulation of sensory receptors, in spite of the fact that mating itself lasts not more than an hour. The mating effect was observed only if the ventral nerve cord remained intact for at least two days after mating. This would indicate that the effect of mating is not based solely on a nervous mechanism. Certainly a humoral factor given off by the sperm mass is not involved, since castrated males may elicit the full response of the corpora allata after mating. One other case that might point to a similar situation was recently reported. In males of *Periplaneta* severance of the ventral nerve cord behind the subesophageal ganglion results in uninhibited activation of the phallic nerves starting 5 to 10 minutes later and increasing for the next 20 min. (Milburn *et al.*, 1960). Here, likewise, "information" carried by nerves does not instantaneously cause a result.

Nothing is known about the actual mode of transmission of the mating stimuli in other insect species. However, it is reported that in *Periplaneta* mating accelerates egg maturation (Griffiths and Tauber, 1942; Roth and Willis, 1956). Also in *Schistocerca* (Norris, 1954) and in the bed-bug *Cimex* (Mellanby, 1939) mating seems to speed up egg maturation in the ovaries.

Thus in some insects mating is a very important factor controlling the rate of egg maturation, since mating apparently counteracts the inhibition of the corpora allata by the brain. Recent experiments in *Leucophaea* seem to indicate that the last abdominal ganglion in virgins influences the brain via the ventral nerve cord to inhibit the corpora allata. Severance of the ventral nerve cord in virgins thus cuts off this influence. It may therefore be assumed that the normal mating act exerts its effect first on the last abdominal ganglion that

influences the brain, which in turn ceases to inhibit the corpora allata. However, this might not be the only pathway indicated by this experiment, since normal mating is more effective than the severance of the last abdominal ganglion from the brain. The whole mechanism is probably even more complex than these observations suggest. In *Diploptera*, for example, the severance of the ventral nerve cord has no effect on the corpora allata (Engelmann, 1959). Perhaps there is a species-specific mechanism.

Other factors, like an egg case in the brood sac, may also act on the last abdominal ganglion. This may be concluded from the fact that severance of the ventral nerve cord in pregnant females of *Leucophaea* results in the activation of the corpora allata just as it does in virgins. In this case it is not known whether the egg case in the brood sac acts on the last abdominal ganglion by nervous or humoral factors. Humoral factors from the egg case, which have been shown to influence the corpora allata, may act on the last abdominal ganglion or the brain. There is accordingly no reason to believe that the presence of an egg case in the brood sac is mechanically recorded in the brain (Roth and Stay, 1959). The question is still undecided.

It seems certain, however, that humoral factors can influence the activity of the corpora allata in a number of insects, either directly or indirectly. Mature eggs in the ovaries apparently restrict the activity of the corpora allata, since castration causes hypertrophy of the corpora allata in many species (Thomsen, 1940; Vogt, 1942; Day, 1943; Pfeiffer, 1945; Bodenstein, 1947; Wigglesworth, 1948; Thomsen and Hamburger, 1955; von Harnack and Scharrer, 1956). The restraining influence of mature eggs in the ovaries may not be a specific one, because even muscle homogenate may act in a similar way. Perhaps, then, some metabolite causes the observed restriction of the corpora allata.

One further aspect in the reproduction of *Leucophaea* seems of particular interest: mating behavior. In certain species of cockroaches, including *Leucophaea*, mating behavior consists of a sequence of mutual activities between the sexes (Roth and Willis, 1952). In these species the female moves onto the back of the courting male and feeds on the tergal gland. Unless this happens no copulation occurs. The female seems to be attracted by an odorous substance given off by the male, since after removal of both antennae no mating takes place (Engelmann, 1960). Endocrines were not known to be involved in this type of behavior until recently. After allatectomy the female responds less frequently to courting males, and from this one may conclude that the olfactory threshold for the perception of the male odor normally is lowered by active corpora allata. It seems that only a certain low hormone titer makes the female responsive for the courting male, since no female that had highly active corpora allata was found being mated. The entire mechanism of perception of the male odor and its partial dependence on the corpora allata hormone is still obscure. No general conclusions can be drawn from these observations. In other, even related, species, mating behavior is apparently independent of the corpus allatum hormone.

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OXIDATIVE ENZYMES AND THE INJURY METABOLISM OF DIAPAUSING CECROPIA SILKWORMS*

David G. Shappirio
Department of Zoology, The University of Michigan, Ann Arbor, Mich.

The remarkable control of insect growth by the hormone ecdyson is readily apparent in the events that transform a diapausing Cecropia pupa into a developing adult moth. The pupal diapause, as shown by Williams,[1] is a state of developmental arrest enforced by the failure of the insect's endocrine system to supply the hormonal stimulus required for growth and development. In essence, what the individual diapausing tissues require is ecdyson, secreted by the prothoracic glands. The latter become inactive after pupation and remain so for many months; but when the prothoracic glands recover their secretory function under neuroendocrine stimulation from the brain, the individual tissues once again begin to grow, and commence a sequence of morphogenetic changes that culminates in three weeks' time with the emergence of the adult moth.[1]

One of the principal tasks of present-day insect endocrinology is to seek an understanding of how ecdyson is able to exert its control over the growth process. In molecular terms, one avenue of approach to the problem lies in elucidating the biochemical changes that occur at the termination of diapause in response to the growth-promoting action of ecdyson. The properties of the diapausing insect itself are likewise of interest for understanding the physiological and biochemical adaptations of cells to life in a state of arrested development.

Both these matters were discussed by Williams[2,3] about ten years ago in papers that called attention to the suitability of diapausing and developing silkmoths as subjects for examining biochemical mechanisms in insect growth and metamorphosis. At the time of these papers it was already clear that alterations in oxidative enzymes at the onset and termination of diapause had an important bearing on the problems just mentioned. In particular, and of special interest for the present paper, was the evidence that a synthesis of oxidative enzymes within the pupal tissues represented one type of change promoted by ecdyson in favoring growth. This matter has subsequently been explored in detail in a number of publications.[4-8]

In the present paper I propose to review recent progress in our understanding of respiratory metabolism and respiratory enzymes in relation to the diapause and development of the Cecropia silkworm. After a brief résumé of the metabolic and enzymatic changes during the course of metamorphosis, two principal topics will be considered: (1) the peculiar properties of respiratory metabolism in diapausing tissues and recent changes in our ideas as to their physiological basis; and (2) the close parallel between respiratory changes normally associated with the termination of diapause and those that follow

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localized integumental injury to diapausing pupae. Insight into the biochemical effects of integumental injury has considerably broadened our perspectives on the significance of respiratory changes, in relation to the hormonal control of diapause and development.

Respiratory Metabolism and Metamorphosis

It has been known for many years that the pupal stage in insect metamorphosis is characterized by a U-shaped curve as regards over-all respiratory metabolism.[9] The descending limb of the curve follows pupation and a corresponding rise is correlated with adult development. This phenomenon is well exemplified in lepidopterous species that experience a pupal diapause. In the Cecropia silkworm, as shown by Schneiderman and Williams,[10] the respiration rapidly falls after the pupal molt to a level less than two per cent of that of the same animal prior to pupation. Respiration persists at an extremely low level throughout diapause. Then, when growth and metamorphosis are resumed under the influence of ecdyson, respiration begins a

TABLE 1

RELATIVE CYTOCHROME CONCENTRATIONS IN INDIVIDUAL CECROPIA TISSUES AT SUCCESSIVE STAGES IN METAMORPHOSIS

Stage in metamorphosis	Relative concentration of cytochrome*			
	b	*c*	$a + a_3$	b_5
Larva	++	++	+++	+++
Diapausing pupa	0	0	+	+
Early developing adult	+	+	++	++
Late developing adult	++	++	+++	+++

* Relative concentrations indicated as +++, high; ++, moderate; +, low; 0, not detectable. Observations at each stage based on several of the following: wing epidermis, fat body, heart, mid-gut, gonads, and malpighian tubules. Data from Shappirio and Williams.[7]

rapid rise that continues during the formation of the adult moth. In concurrent studies, Schneiderman and Williams[5] also established in Cecropia that the scant respiration of the diapausing pupa is relatively insensitive to cyanide and carbon monoxide, in concentrations that markedly suppress the respiration of the pre- and postdiapausing insect.

The Cytochrome System and Metamorphosis

Additional insight into these matters has been provided subsequently through studies of the respiratory enzymes themselves. By spectroscopic methods, based on the low-temperature technique of Keilin and Hartree,[11] it was possible to characterize conspicuous alterations in the cytochrome system of individual Cecropia tissues during the course of metamorphosis.[7] These observations are illustrated in TABLE 1, which summarizes changes occurring in epidermis, fat body, heart, mid-gut, gonads, and malpighian tubules. As shown in TABLE 1, prior to the pupal diapause the rapidly growing larval tissues contain moderate to high concentrations of cytochromes *b*, *c*, $a+a_3$, and b_5. In contrast, the tissues of the diapausing pupa show a peculiar cytochrome

system in that components *b* and *c* are not detectable spectroscopically. Cytochromes $a+a_3$ and b_5 persist in low, although detectable, concentration. Several months later, when growth and metamorphosis are resumed in response to ecdyson, cytochromes *b* and *c* reappear in detectable concentration. Together with $a+a_3$ and b_5 they then undergo a progressive increase in concentration during the course of adult development.

It is worth noting at this point that the intersegmental muscles of the insect retain a normal cytochrome system, containing moderate titers of components *b*, *c*, and $a+a_3$ throughout diapause, as well as in the pre- and postdiapausing insect. Apparently the somatic muscles do not participate in the changes just described. Recent studies (Shappirio, unpublished) show that the pupal brain also retains detectable concentrations of cytochromes *b*, *c*, and $a+a_3$ throughout diapause. Information on the status of the cytochrome system in the pupal brain has not hitherto been available.

However, for epidermis, fat body, heart, gut, gonads, and malpighian tubules, one can summarize the spectroscopic studies as giving evidence for a marked breakdown of cytochromes after pupation and an equally prominent resynthesis associated with the termination of diapause. These conclusions have been strengthened greatly by the finding[8] that several oxidative enzyme systems, including DPNH oxidase, DPNH-cytochrome *c* reductase, succinate-cytochrome *c* reductase, and cytochrome *c* oxidase, show alterations in activity, coupled with the onset and termination of diapause, which essentially parallel the spectroscopic changes described above.

Let us consider the implications of these conclusions in relation to the respiratory metabolism of the diapausing insect. The best way to view cellular respiration is in terms of its role as a chemical energy transformer to provide high energy phosphorus and sulfur compounds for meeting the endergonic demands of living cells. Pupal respiration is exceedingly low; but the Cecropia pupa is nevertheless an obligatorily aerobic organism that depends on respiration in order to exist.[12] In the absence of extensive muscular activity we can probably generalize and say that the principal energy requirements of the pupa are for synthetic reactions and active transport processes. In relation to the former, several workers have shown that the incorporation of labeled precursors into proteins and nucleic acids, and presumably their synthesis, continue during diapause but at a low level.[13-15] If one regards cytochrome concentrations as reflecting the metabolic requirements of the tissue,[16] then the characteristics of the cytochrome system in pupal tissues can be viewed as an adaptation to life in a state of arrested development.

Terminal Oxidative Metabolism in Diapausing Tissues

The over-all respiration of the diapausing pupa is interesting not only because of its extremely low rate, but also because of its relative insensitivity to inhibitors of cytochrome oxidase such as carbon monoxide.[5] When the latter inhibits respiration, and particularly if this inhibition is reversed by light, one may conclude with reasonable certainty that the respiration is mediated via the classical cytochrome *c* oxidase pathway ($a+a_3$). However, when carbon monoxide fails to show a large inhibitory effect even at relatively

high CO/O_2 ratios, a conclusion regarding the terminal oxidative pathway is commonly rendered much more difficult. Such has been the case with diapausing silkmoth pupae, as with a variety of plant and animal tissues showing sensitivity to the inhibitor.

Insensitivity to carbon monoxide would arise if respiration were mediated by a terminal oxidase, other than $a+a_3$, and capable of functioning in the presence of the inhibitor. Some years ago it appeared that the CO-resistant properties of pupal respiration could be best explained in terms of such a different terminal oxidative pathway.[5] The most promising candidates for the role of terminal oxidase appeared to be an autoxidizable flavoprotein or cytochrome.

More recently, however, experimental evidence has been obtained[17,18,31] in favor of an alternative explanation of pupal respiratory metabolism: namely, that the respiration is mediated by cytochrome oxidase itself. This explanation rests on the theory that cytochrome oxidase-mediated respiration becomes insensitive to carbon monoxide under certain conditions. These conditions demand, principally, that oxidase be present in large excess in relation to the actual rate of electron transport (that is, respiratory activity). Under such conditions the oxidase may be regarded as relatively "unsaturated" by electron transfer; even at high concentrations of carbon monoxide, the equilibrium between oxidase and inhibitor probably affords sufficient free oxidase to satisfy the relatively low respiratory requirements.

Although evidence for this theory has been available for a number of years,[19,20] its applicability in the case of diapausing Cecropia pupae became apparent only relatively recently[8] upon the realization that conditions in most pupal tissues were probably of just the right sort to permit the function of cytochrome a_3 as an "insensitive" oxidase. As will be recalled, in most pupal tissues cytochromes $a+a_3$ are present while cytochromes *b* and *c* are below a detectable concentration. Moreover, the respiration is extremely low.

The principal published evidence on diapausing pupae is that of Harvey and Williams[17] for the pupal heartbeat and that of Kurland and Schneiderman[18] for the pupa as a whole. As shown by these workers, heartbeat and respiration are not affected by lowering the partial pressure of oxygen to that of a few millimeters of mercury. But under these conditions both processes become sensitive to carbon monoxide. At the low oxygen pressure, turnover of cytochrome oxidase is presumably rate-limiting, and the great excess of oxidase is removed. These experiments provide a persuasive argument that pupal heartbeat and respiration are mediated by cytochromes $a+a_3$ and not by some alternative oxidase.

If cytochrome oxidase is present and functioning in the diapausing tissues, then one is obliged to inquire as to the whereabouts of cytochrome *c*, which is undetectable spectroscopically.[7] Since cytochrome *c* is the only known substrate for cytochrome oxidase, at least *in vivo*, the simplest explanation is that *c* is actually present in the pupal tissues, but not in detectable concentration.

This analysis of respiratory metabolism in diapausing tissues leads to several important conclusions. In the first place, it signifies that the CO-resistance of the pupa conceals a fundamental dependence of respiration on cytochrome oxidase. Since the pre- and postdiapausing tissues likewise depend on cyto-

chrome oxidase-mediated respiration, it persuades one to conclude[18] that the respiratory metabolism shows quantitative variations during metamorphosis, but not qualitative changes as had hitherto seemed probable.[5]

One would also predict, on the basis of the analysis described above, that the primary determinant for sensitivity to carbon monoxide is the magnitude of respiratory electron transfer in relation to the potential turnover of the available cytochrome oxidase. When the subterminal electron transfer proceeds rapidly and substantially saturates the oxidase, sensitivity is high. Otherwise the effect of carbon monoxide is low, even though cytochrome oxidase mediates the respiration that occurs. This analysis is supported particularly well in studies, to be described later in this paper, dealing with the CO-sensitivity of pupae whose respiratory metabolism is accelerated after integumental injury.[18,31]

Respiratory Metabolism at the Termination of Diapause

We may now return to the events that occur when pupal diapause is terminated, in response to the action of ecdyson. At this time, cytochromes *b* and *c* reappear in spectroscopically detectable titer within the individual tissues, and together with $a+a_3$ and b_5 commence a rise in concentration that accompanies adult development. Actually the onset of these changes can be detected several days prior to the first morphological sign of development in terms of increased activity of several oxidative enzyme systems.[8]

Simultaneously the respiratory metabolism begins a conspicuous rise and becomes progressively more sensitive to carbon monoxide.[5,10] According to present views, and as stated by Kurland and Schneiderman,[18] the increased sensitivity reflects a correspondingly enhanced saturation of cytochrome oxidase.

Schneiderman and Williams have also shown[5] that when carbon monoxide is applied to pupae just prior to the outset of adult development, respiration fails to rise and the insect remains in diapause. And if the gas is applied to early postdiapausing animals, during the first few days of adult development, respiration is suppressed to about the diapausing level. Developmental progress then ceases. These experiments show that function of the normal cytochrome oxidase system, at rates above that in the diapausing pupa, is essential for the termination of diapause. One can conclude that cytochrome synthesis, and the enhanced respiratory metabolism that it permits, are prerequisites for the insect's developmental response to ecdyson.[5]

In the normal course of events, cytochrome synthesis is inextricably associated with adult development, and it is difficult to determine how closely the cytochrome synthesis is related to the primary action of ecdyson. As has been stated elsewhere, the changes in the cytochrome system at the termination of diapause may be typical of a number of synthetic processes, all required for development, but which occur following some more fundamental action of ecdyson upon the diapausing cell.[15,21] In order to resolve this question, one might seek a means of dissociating the cytochrome synthesis from the termination of pupal diapause and the developmental response. Such an opportunity has to a large extent been provided by studies on the respiratory enzymes of pupae receiving localized integumental injury.

Injury Metabolism

During the course of their metabolic studies, Schneiderman and Williams[10] confirmed for Cecropia that localized injuries cause a conspicuous rise in the animal's respiration. This enhanced respiratory metabolism is termed the "injury metabolism." The magnitude and duration of the injury metabolism were found to depend on the extent of injury. Large injuries, involving the removal of sizable areas of pupal integument, cause a rise in respiration that continues for several days, until the metabolism equals or exceeds that of the postdiapausing insect. Respiration remains at the high level for many weeks and then gradually declines, reaching the initial level only after several months.

It is of special interest and importance to note that although metabolism attains a pace that equals or exceeds that when diapause is terminated, injury is followed by no over-all development and, in fact, no morphogenetic response save for a healing of the epidermal wound.[10] This healing response is localized; yet the metabolic changes of the animal are far too large to be accounted for solely in terms of an activated metabolism in the localized area of wounding.

The injury metabolism is characteristic only of diapausing pupae.[12] Considerable evidence argues that it is not attributable to muscular activity and that the metabolic response does not depend on the insect's central nervous system.[10,12] Moreover the injury metabolism can be obtained in isolated pupal abdomens devoid of the known endocrine organs.[10,12] It appears to be generalized metabolic response on the part of the individual diapausing tissues.

The effect of carbon monoxide on the injury-stimulated respiration is a matter of particular interest. According to the hypothesis presented earlier, the degree of sensitivity to this inhibitor will rise as increasing respiratory rate provides a greater "saturation" of cytochrome oxidase. Thus, diapausing pupae receiving only very small injuries, and showing only a relatively slight increase in respiratory metabolism, would be expected to show a sensitivity to carbon monoxide only slightly greater than in the case of uninjured pupae. Large injuries, on the other hand, should lead to a greatly increased sensitivity, in keeping with the greater enhancement of respiratory rate. On the basis of considerable experimental evidence, treated elsewhere,[24,18,31] it is possible to conclude that the above view is substantially correct. Sensitivity to carbon monoxide is a function of respiratory rate.

The latter conclusion applies not only to injured pupae, but, according to this evidence, to uninjured pupae and to developing adults. On the basis of inhibitory studies, one is drawn ever closer to the view that respiration in Cecropia shows a fundamental dependence on cytochrome oxidase throughout pupal diapause and adult development.

For the pupa as a whole, the alterations in respiratory metabolism after injury strikingly parallel those that accompany the termination of diapause. Therefore it has been a matter of considerable importance to determine whether the over-all metabolic effects of injury are accompanied by alterations in the cytochrome system in individual tissues. We may now consider several series of experiments designed to provide an insight into this question. A preliminary account of these experiments has already been published,[22] and they will be considered in detail elsewhere.[31] The studies are based on diapausing pupae

in which a large integumental injury was established by excising cuticle and epidermis from the facial and leg region. Routine surgical techniques, developed by Williams for silkmoth pupae, were utilized; and the wounded area was covered by a previously shaped piece of cellulose acetate sealed in place with melted paraffin wax.[23]

Such an injury is routinely followed, as shown in FIGURE 1, by an immediate doubling of respiration and a subsequent rise that persists for several days. In the case of the pupa shown, the respiration leveled off at about seven times that measured prior to the injury. The metabolic rate of animals receiving

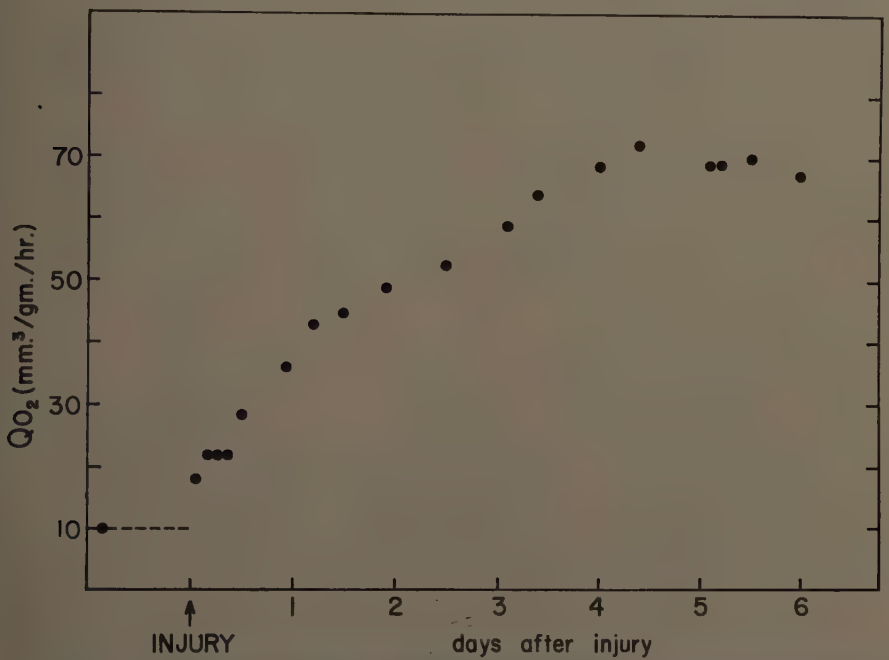

FIGURE 1. The oxygen uptake of a diapausing Cecropia pupa after a large integumental injury. The injury involved removal of cuticle and epidermis in the facial and leg region.

the large injury is quantitatively equivalent to that of animals at the outset of adult development.

Principal efforts were directed toward a single tissue, the wing epithelium; and to the measurement of several oxidative enzyme systems, including DPNH oxidase, DPNH-cytochrome *c* reductase, succinate-cytochrome *c* reductase, and cytochrome *c* oxidase. Considerable information on the behavior of these enzyme systems in wing homogenates was already available; measurements of their activities at successive stages in pupation, pupal diapause and adult development had been utilized[8] to provide quantitative confirmation of the spectroscopic observations discussed earlier.

TABLE 2 permits one to observe the effect of large integumental injury on the activities of oxidative enzyme systems in the wing. The upper horizontal row of figures shows activities encountered in wing preparations from uninjured

diapausing pupae. In the second row of figures are illustrated the activities in wings of pupae from the same batch of diapausing animals, but prepared for analysis one week after injury. If one compares the uninjured and injured pupae, it is readily apparent that the latter reveal a detectable level of DPNH oxidase activity whereas this activity is not detectable in wings prior to injury. The appearance of detectable DPNH oxidase activity involves more than a twofold increase in activity as expressed on a nitrogen basis. DPNH-cytochrome *c* reductase, succinate-cytochrome *c* reductase, and cytochrome *c* oxidase likewise show a two- to threefold increase in activity following the injury.

The levels of oxidative activity encountered in wings from injured pupae are of the same order of magnitude as those observed in animals just after the termination of pupal diapause. This will be evident from the third line of

TABLE 2

EFFECTS OF INTEGUMENTAL INJURY ON THE ACTIVITY OF OXIDATIVE ENZYME SYSTEMS IN THE WING EPITHELIUM

Type of animal	Number of experiments*	DPNH oxidase	DPNH-cytochrome *c* reductase	Succinate-cytochrome *c* reductase	Cytochrome *c* oxidase
Uninjured diapausing pupae	6	<3	66	24	300
Pupae one week after injury	6	7	140	47	910
Animals at outset of adult development	4	11	114	48	1500
Pupae six months after injury	3	<5	80	18	290

* In each experiment, the wings from six insects were pooled, and then homogenized and prepared by differential centrifugation, as described elsewhere,[8] to yield a combined mitochondrial-microsomal suspension for spectrophotometric assay of each of the enzyme systems. The activities are averages for all experiments, and are expressed on a nitrogen basis, namely: DPNH oxidase, as millimicromoles DPNH oxidized/mg. N/min.; DPNH- and succinate-cytochrome *c* reductase, as millimicromoles cytochrome *c* reduced/mg. N/min.; and cytochrome *c* oxidase, as millimicromoles cytochrome *c* oxidized/mg. N/min. When DPNH oxidase activity was below a detectable level, the table records "less than" the minimum activity that would have produced a detectable change in absorption.

TABLE 2, which illustrates activities in wings from animals on the "zero" to second day of adult development.* Except possibly in the case of cytochrome *c* oxidase, it is essentially impossible to distinguish between wings from injured pupae and those from animals at the outset of adult development.

Six months after injury, pupae belonging to the same batch that was utilized earlier had recovered from the injury metabolism, in that their respirations had declined to levels characteristic of uninjured diapausing pupae. TABLE 2 illustrates that oxidative activities in wing preparations also declined. DPNH oxidase activity was found to be undetectable, and the other enzyme systems exhibited activities substantially similar to those encountered prior to injury. This "recovery" of oxidative enzyme systems is summarized graphically in FIGURE 2, which illustrates DPNH-cytochrome *c* reductase, succinate-cytochrome *c* reductase, and cytochrome *c* oxidase activities plotted relative to the preinjury level for uninjured, injured, and "recovered" individuals. In the

* Stages in adult development were ascertained according to criteria of Schneiderman and Williams.[6]

case of each enzyme system, one can conclude that injury causes a two- to threefold increase in activity within a week's time, and that the activities subsequently decline to about the preinjury level.

The above experiments reveal a doubling or tripling in enzymatic activities one week after injury, when the activities are calculated on a nitrogen basis. In actual fact, the nitrogen content of the wing preparations itself shows an approximate doubling after injury. Consequently the actual increase in absolute activity is four- to sixfold. In the present case it seems likely that this increase in activity is associated at least in part with an actual synthesis of oxidative enzymes within the wing. By spectroscopic means, one can observe an increased concentration of cytochromes b_5 and $a+a_3$ in wings from injured pupae, and in favorable preparations it is also possible to observe a detectable absorption band of cytochrome *c*. Unfortunately, the technique of low-temperature visual spectroscopy utilized does not afford a quantitative insight into the magnitude of the increase in cytochrome concentration.

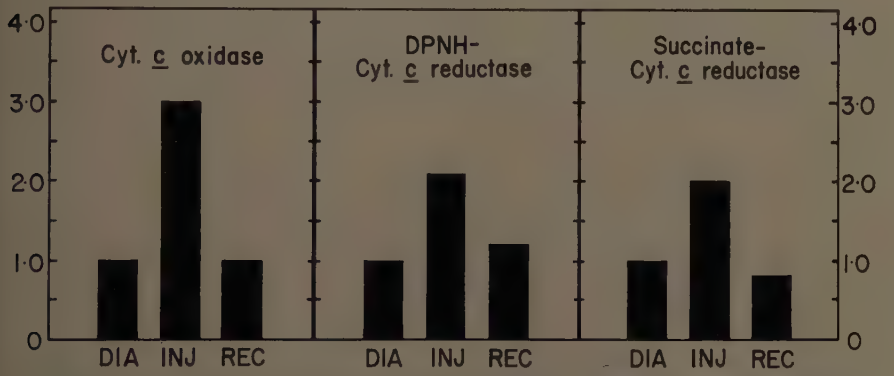

FIGURE 2. Activities of oxidative enzyme systems in uninjured diapausing pupae (DIA), pupae one week after injury (INJ), and "recovered" pupae six months after injury (REC). The activities are shown relative to the preinjury level. Data from TABLE 2.

A body of evidence is now accumulating that suggests that the oxidative changes just described are accompanied by equivalent enhancement of a variety of endergonic processes within the pupa. It has been known for some years from the work of Telfer and Williams[13,14] that the rate of incorporation of C^{14}-labeled amino acids into pupal blood proteins is low, but that it is augmented after injury by about the same extent as the increase in respiratory metabolism. And in the wing itself, G. R. Wyatt (personal communication) has observed that the incorporation of radioactive phosphate into ribonucleic acid is also enhanced five- or sixfold after injury.

It is important to emphasize that these biochemical changes parallel those normally occurring when diapause is terminated. Indeed, on the basis of the biochemical criteria thus far studied in Cecropia, one is virtually unable to distinguish injured pupae from animals at the outset of adult development.

However, as stated earlier, the injured pupa fails to reveal any of the morphogenetic changes that characterize the onset of adult development in response to ecdyson. The wings of injured pupae do not begin to differentiate;

nor do the spermatogonia or spermatocytes within testes of male pupae show any visible changes after injury, as demonstrated by Williams (personal communication), although their prominent differentiation into spermatozoa is one of the earliest signs of adult development. It seems necessary to conclude that cytochrome synthesis and accelerated incorporation of precursors into pupal protein and nucleic acid are not sufficient in themselves to permit the termination of diapause.

Injury, Wound-healing, and Hormones

The only morphogenetic response thus far observed in injured Cecropia pupae is localized: a healing of the epidermal wound. Certain information about the wound-healing process is already available.[25-27] Under the plastic window the wound is first healed by a thin membrane formed from blood cells that migrate to the wounded site; the area is then invaded by ingrowing epidermal cells and tracheoles that derive from the surrounding tissue. According to Smith and Schneiderman,[27] the healing process is unaccompanied by mitotic activity. Schneiderman[6] has also demonstrated that wound-healing is prevented by exposure of the animal to carbon monoxide, or by prior injection of diphtheria toxin; these experiments show that the wound repair in itself depends on oxidative metabolism and imply that cytochrome synthesis is required.

Healing of epidermal wounds is, presumably, a desirable process for the pupa. But what is of particular interest in the present context is that seemingly the biochemical resources of the animal as a whole are mobilized in response to localized injury. Apparently in Cecropia an "injury factor," released from the wounded site, is of importance in evoking the generalized metabolic response; injury to one member of a pair of pupae joined in parabiosis causes an injury metabolism in the other pupa.[12,28]

Insect endocrinologists will recognize a familiar concept in this idea of an "injury factor," and, more particularly, in the similarity between events occurring after injury and those that normally accompany growth. As long ago as 1937, Wigglesworth[29] pointed out that the localized changes following small epidermal injuries to *Rhodnius* show a striking similarity to events in the early stages of normal growth and molting. Wigglesworth concluded that a diffusible factor, perhaps a product of autolysis in the injured region, somehow activated the surrounding cells. He has subsequently[30] continued a detailed cytological study of wound repair in *Rhodnius*. Wigglesworth makes it quite clear that the changes after injury proceed in the absence or inactivity of the brain and thoracic glands. He re-emphasizes that the injury factor has the same effect as ecdyson in the localized area of wounding; and the possibility remains open that the factor may even be ecdyson itself. The latter conjecture is, of course, exceptionally intriguing, since it implies that tissues other than the thoracic glands may be capable of producing ecdyson and that the endocrine function of these organs represents the specialized production of, as expressed by Wigglesworth, "some widespread metabolite." However, in so far as the Cecropia silkworm is concerned, we have no evidence for the presence of ecdyson, at least in concentrations capable of eliciting a morphogenetically detectable response in the direction of adult development.

Conclusions

Studies on the injury metabolism of diapausing silkworms have provided us with a clearer perspective on several important matters. First, they have helped provide an insight into the probable basis of the respiratory insensitivity to carbon monoxide that characterizes diapausing pupae. According to present views, respiration is mediated by cytochrome oxidase, and the degree of saturation of the oxidase by subterminal electron transport is a prime determinant of sensitivity to the inhibitor. This permits us to conclude that despite major alterations in respiratory metabolism and sensitivity to inhibitors, respiration of the Cecropia silkworm depends fundamentally on cytochrome oxidase at all stages of the life history. Studies of injury metabolism also permit us to draw certain conclusions regarding the relationship between cytochrome synthesis and the action of ecdyson in terminating pupal diapause. As we have already seen, it appears that this synthesis, although a prerequisite for adult development, is not in itself sufficient to permit the initiation of adult development. It is clear that growth and metamorphosis require energy supplies above and beyond the requirements for maintenance of the diapausing state. But the provision of this energy supply is not enough to evoke growth and metamorphosis.

Acknowledgments

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HORMONAL CONTROL OF CASTE DIFFERENTIATION IN TERMITES*

Martin Lüscher
Division of Animal Physiology, Zoological Institute, University of Bern, Bern, Switzerland

Introduction

Caste differentiation has been studied in the European dry wood termite *Kalotermes flavicollis* (Fabr.). In this termite species three fixed castes are known: soldiers; alates (winged imagos or adults), which later become primary reproductives (king and queen); and secondary or supplementary reproductives. The normal development, shown in FIGURE 1, leads through about six to eight larval stages† to the stage of the full-grown larva or pseudergate (Grassé and Noirot, 1947; Lüscher, 1952). In these pseudergates growth is arrested while the molting cycles remain undisturbed. The pseudergate can make any number of stationary molts. Alternatively it may pass through two nymphal stages to the winged adult, through a presoldier or white soldier to a soldier, or directly to a supplementary reproductive. Soldiers and supplenentary reproductives usually develop from pseudergates, but they can also arise from either of the two nymphal stages or from larvae of the fifth stage onwards. Both nymphal stages can undergo regressive molts, becoming pseudergates again. The pseudergate has at each molt four differentiation possibilities: it can molt without growing and remain a pseudergate; it can differentiate in adult direction and become a wing-padded nymph; it can change into a supplementary reproductive; or it may produce a white soldier or presoldier.

These four differentiation possibilities and their hormonal control will now be discussed in the light of recent investigations, which have in part been carried out with A. Springhetti of the University of Pavia, Pavia, Italy.

The Endocrine Glands of Kalotermes flavicollis

The endocrine glands are shown in FIGURE 2. The neurosecretory cells in the brain, the corpora cardiaca, and the corpora allata are not much different from those of roaches. The hypocerebral ganglion is probably also an endocrine gland. It seems to vary regularly in size in different castes and developmental stages, but it has thus far not been studied in detail. The prothoracic glands are composed of two parts. One part, which is entirely in the head, consists of lobulated strands of small cells, which run along the muscles connecting the esophagus with the tentorium. Some lobes of the gland are in close contact with the corpora cardiaca. This part of the gland has been described as tentorial gland by Jucci, who as early as 1924 suggested that it is a gland of internal secretion. It has been redescribed under the name of ventral gland by Pflugfelder (1947). The second part of the prothoracic glands con-

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† It is in this case convenient to use the German and French terminology: larvae for stages without wing pads; nymphs for stages with wing pads.

sists of two long and very thin strands of cells deviating from the first part, running parallel to the connectives of the ventral nerve chain into the thorax and ending near the prothoracic ganglion. These strands are probably homologous to the crossed cell strands of the prothoracic glands of roaches, as described by Scharrer (1948) for *Leucophaea*, although they contain no musculature. Both parts of the gland form a histological unit, and the same cyclical changes occur simultaneously in both parts. We therefore consider the whole complex as the prothoracic gland. As in other insects, the prothoracic glands degenerate in the winged adult after metamorphosis. They also degenerate in supplementary reproductives, but they outlast in soldiers, although these, like adults, are unable to molt again.

Stationary Molts

Normally, in small laboratory colonies only stationary molts are observed. Nymphal development occurs when the colony becomes larger, probably when

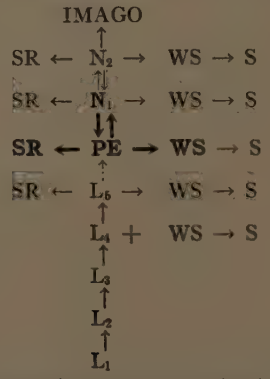

FIGURE 1. Course of development in *Kalotermes flavicollis*. The arrows indicate molts, the broken line the occurrence of several molts. L = larvae; N = nymphs; PE = pseudergate; WS = white soldiers (presoldiers); S = soldiers; SR = supplementary reproductives.

the nutritional conditions are better. This is observed in nature, where stationary molts seem to occur mainly in spring, when many young larvae and wing-padded nymphs have to be fed by the pseudergates.

Histological investigations show that the corpora allata volume increases soon after each molt and reaches a maximum about 10 to 15 days after the molt. It then decreases, but increases again about 15 days before the next molt. The first maximum probably has nothing to do with the determination of a stationary molt. Its possible significance will be discussed later. The second maximum, however, which lies in the premolting period, suggests a juvenile hormone secretion that may be responsible for preventing the appearance of nymphal characters at the molt. Nevertheless, juvenile hormone seems to be present in large enough quantities throughout the intermolt period, since inducing molts prematurely by injecting the molting hormone "ecdyson" had no effects upon differentiation (Lüscher and Karlson, 1958).

Nymphal and Imaginal Differentiation

It is exceptionally rare for nymphs to develop in well-established small laboratory colonies. As the fate of marked individuals is very difficult to

follow in large colonies, almost no exact data on nymphal development are available. For the same reason no volume measurements of the corpora allata have been made during nymphal development. In nature first-stage nymphs develop in late summer, just before or after the time when the adults are swarming. The pseudergates then no longer have to feed many other individuals and general nutritional conditions are probably good. If second-stage nymphs

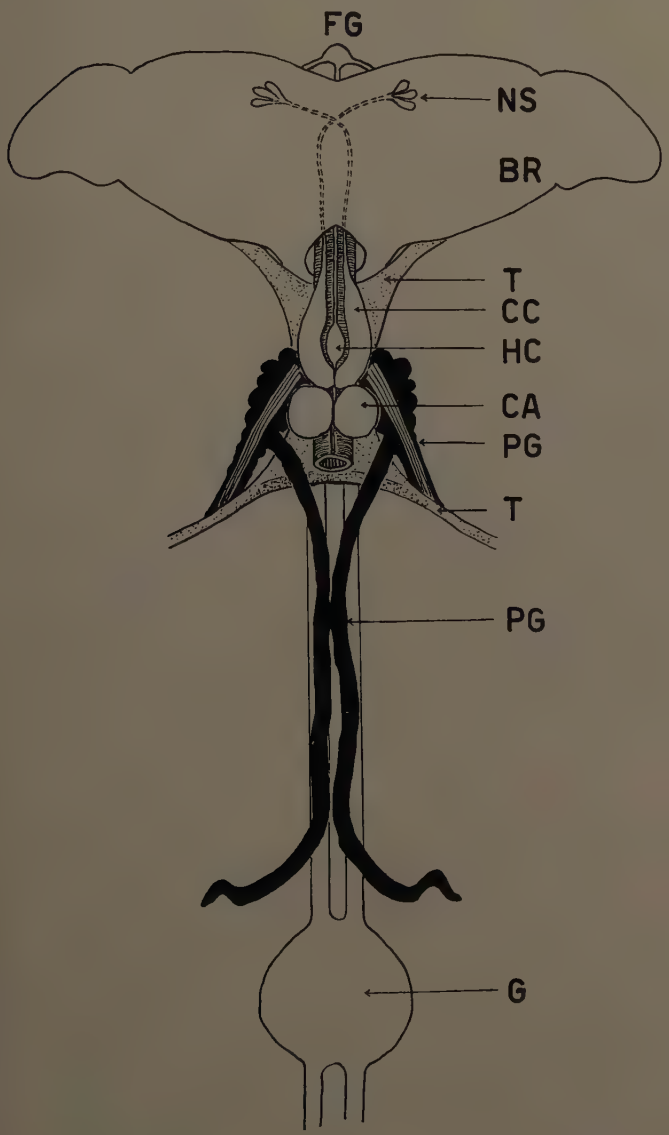

FIGURE 2. Endocrine system of *Kalotermes flavicollis*. FG = frontal ganglion; BR = brain; NS = neurosecretory cells; T = tentorium; CC = corpora cardiaca; HC = hypocerebral ganglion; CA = corpora allata; PG = prothoracic glands; G = first thoracic ganglion.

are transferred from large into small laboratory colonies before a critical period, they undergo regressive molts (Lüscher, 1952). In the small colony, with less trophallactic exchange possibilities, nutritional conditions are probably generally worse. This situation, and the observations made in nature suggest that nutrition may be an important factor in the determination of nymphal development.

In so far as hormonal control of nymphal differentiation is concerned, we may postulate, in analogy to the results obtained in other insects by various authors, that a decreasing activity of the corpora allata is responsible for nymphal differentiation and a complete inactivation of these glands in the last nymphal stage for adult differentiation. It therefore seems likely that transferring nymphs from large into small colonies will prevent the inactivation of the corpora allata if it has not occurred already before the time of transfer.

By injecting the molting hormone "ecdyson"* into second stage nymphs it is possible to obtain a whole series of intermediate forms, ranging from nymphs with slightly longer wing pads and larval genitalia to slightly nymphoid adults (FIGURE 3). There are two possible explanations for this: (1) the juvenile hormone titer normally may be decreasing gradually during the nymphal stage, so that at the time of the injections different amounts of juvenile hormone were present and prevented adult differentiation more or less effectively; (2) it may be that in these nymphs adult development had already started when molting was induced artificially, and that the time available was insufficient for terminating the adult differentiation before the molt occurred. In that case it may be assumed that a slight activity of the prothoracic gland is responsible—in the absence of juvenile hormone—for the initiation of adult differentiation, and that the ecdyson titer necessary for the induction of the molting process is normally reached only when differentiation is so advanced that it can be completed before the molt.

The second explanation for the occurrence of intermediate forms is more likely to be true for various reasons. After the transfer of second-stage nymphs from large into small colonies, intermediate forms are never produced. This indicates that at the time of transfer the corpora allata were already inactivated completely or were still being active. The inactivation of the corpora allata therefore takes place rather suddenly, as in the roach *Leucophaea*, where histological studies revealed that the corpora allata are inactivated rather suddenly six or seven days after the last nymphal molt (Lüscher and Engelmann, 1960). It is only up to that time that extranymphal instars can be produced by an extensive injury or by cutting the corpus allatum nerves, thus keeping the corpora allata active. Intermediate forms never occurred in these experiments. Scharrer (1946) showed that in the same roach the critical period for influencing the development of nymphs by removing the corpora allata is relatively early in the molting interval; also in this case no intermediate forms were produced. These results suggest that in roaches differentiation begins before the molt is induced and that molting is induced only when the type of differentiation is fully determined.

In termites the corpora allata have little or no effect after the induction of

* I am indebted to Peter Karlson of the Max Planck-Institut für Biochemie, Munich, Germany, for supplying a sample of the molting hormone "ecdyson."

the molting process. This has been shown in ligation experiments, in which the head was separated from the thorax before pseudergate molts just after the emptying of the gut (this occurs seven days before the molt and is the first effect of the molting hormone after its release). The ligated termites showed no sign of nymphal development in the absence of the corpora allata.

There is one phenomenon in termites that demonstrates even better the fact that differentiation begins well before molting is induced. Grassé (1949) has observed in normal orphaned colonies of *Kalotermes* the rather rare occurrence of an intermediate form between nymph and adult, the so-called pseudimago, which is functioning as a supplementary reproductive. We have observed such forms several times in our laboratory colonies. One of them is shown in FIGURE 4. If we compare this pseudimago with the intermediate forms obtained after an injection of ecdyson, we find that, according to the size of the

FIGURE 3. Intermediate forms between nymphs and adults, produced experimentally by injecting ecdyson. Magnification ×10.

wings, it is comparable to a slightly adultoid nymph (FIGURE 3*a*) but that, according to the mode of attachment of the wings to the corresponding thoracic segments, it is comparable to the nymphoid adults as shown in FIGURE 3*c*. This indicates that this nymph had undergone a differentiation in adult direction up to the point at which the attachment of the wing was differentiated. Then a reversal of differentiation with a regression of the wing size must have taken place, probably under the influence of a renewed activity of the corpora allata. Under normal conditions, this regression would have been completed before the molt but, after orphaning the colony this individual again changed its differentiation to become a supplementary reproductive and, in these, as we shall see later, the molting process is initiated immediately after determination. At this prematurely induced molt, then, the state of dedifferentiation reached at that time became manifest.

Thus it is probable that under normal conditions pseudergate, nymphal, or

adult differentiation sets in quite early in the molting interval. It may for some time switch over to another type of differentiation, according to the juvenile hormone titer. The differentiation process is probably initiated by a slight activation of the prothoracic glands. Molting itself is induced only when differentiation is well advanced in one or the other direction, so that it can be completed before the actual molt occurs. The induction of the molt in termites therefore is a part of the differentiation process; there must be a mechanism responsible for timing the molt, depending on the state of differentiation. This accounts for the enormous variation in the duration of the molting interval (50 to 200 days; Lüscher, 1952). It also accounts for the fact that intercastes are extremely rare.

These results are in contrast to the findings in *Rhodnius* (Wigglesworth,

FIGURE 4. Pseudimago, a rarely-occurring intercaste between supplementary-reproductive and adult. Magnification ×10.

1954), in which differentiation sets in after the induction of the molt. It seems probable that the bloodsucking bug *Rhodnius*, with the infallible dependence of its molting cycles on blood meals, is a special case in that respect.

An intriguing problem is the question of how nutritional factors influence the corpora allata activity in termites. It can be observed in nature that first- and especially second-stage nymphs have an extremely well-developed fat body. Engelmann (1957) has shown that in adult females of *Leucophaea* the corpora allata are inhibited during pregnancy by a substance given off from the eggs in the brood sac. Implanted eggs and even oöcytes, which at ovulation had been retained in the ovaries and were being resorbed there, had the same inhibitory effect upon the corpora allata by a stimulation of inhibitory centers in the brain. It is conceivable, therefore, that as a consequence of good nutrition a metabolic factor, possibly a product of protein breakdown,

might be responsible for the inhibition of the corpora allata, which brings about nymphal and adult differentiation in termites.

Supplementary Reproductive Development

If one or both reproductives are removed from a colony, that is, if the colony is orphaned, any pseudergates at the reactive stage (competence) become determined and develop into supplementary reproductives. Thus the reproductives present in the colony have an inhibitory influence upon supplementary reproductive differentiation. The results of various experiments, including extract feeding experiments, suggest that the inhibition is exerted by the so-called social hormones or pheromones (Karlson and Lüscher, 1959), which are given off by the reproductives and which are distributed throughout the colony by the pseudergates (Lüscher, 1956).

The molting interval before a supplementary reproductive molt is on the average only about one half as long as that before a pseudergate molt. The supplementary reproductive molt must therefore be induced prematurely. When a colony is orphaned, the first pseudergates, which will develop into supplementary reproductives, will empty their guts 24 hours after the removal of the sexual pair and will molt 5 days later. Within the first 24 hours, therefore, determination must have occurred, and the prothoracic glands must have been activated by a release of brain hormone (neurosecretory material). The molting process is much quicker than in pseudergate molts, where the actual molt occurs only 7 days after the emptying of the gut.

In order to test the possibility that a prematurely induced molt brings about supplementary reproductive development, we have induced molts artificially by injecting the molting hormone ecdyson (Lüscher and Karlson, 1958). The injected pseudergates molted nine to ten days later, but they remained pseudergates, even when the dose was four times higher than necessary for inducing a molt.

Histological investigations have shown that the corpora allata begin to increase 5 days before a supplementary reproductive molt, that is, one day after determination. They reach a maximum at the time of the molt or shortly afterwards. It seems, therefore, that this activation of the corpora allata might have something to do with differentiation. In order to test this possibility we implanted corpora allata from freshly molted supplementary reproductives into pseudergates. The implants had no effect upon supplementary reproductive development, but most of the operated termites molted two to six weeks later into presoldiers. This effect will be discussed later. We then tested the possibility of a combined effect of ecdyson and juvenile hormone activity by implanting corpora allata and inducing the molt at the same time by an injection of ecdyson. All termites so treated made normal pseudergate molts.

We then became doubtful about the role of the corpora allata in supplementary reproductive differentiation. We therefore tried to investigate the significance of the sudden increase in volume of the corpora allata by ligating the heads of pseudergates that were expected to develop into supplementary reproductives. The ligations were made just after the emptying of the gut,

that is, before the growth of the corpora allata. Five days later the old skin was removed. The genitalia were those of normal supplementary reproductives. The corpora allata therefore are not necessary for the differentiation of supplementary reproductives. Dissection of the ligated termites, however, showed that the oöcytes had not grown, as they do before a normal supplementary reproductive molt. Thus the sudden growth of the corpora allata after the determination of supplementary reproductive differentiation is probably correlated to a production of gonadotropic hormone.

As the corpora allata have no significance for the determination of supplementary reproductive development and as ecdyson only induces molts, there remains the possibility that the brain hormone itself, which must be produced suddenly and in large quantities, is responsible for inducing supplementary reproductive differentiation. We think that the brain hormone is released in large quantities, because the molting process is accelerated and the molt occurs two days earlier after the emptying of the gut than a pseudergate molt. The hormone of the prothoracic gland cannot be responsible for this acceleration, since even the highest doses of ecdyson did not bring about a significant acceleration of the molting process. There is also histological evidence that suggests a massive release of brain hormone. In differentiating supplementary reproductives the hemocytes are full of vacuoles about two to three days before the molt, and these vacuoles contain material that stains deep blue-black with Gomori's chrome-hematoxylin. It is conceivable that at this stage the hemocytes take up an excess of neurosecretory material from the hemolymph.

The hypothesis that brain hormone is the responsible factor in supplementary reproductive differentiation has not been proved experimentally thus far, but there is one other fact that may support the theory. Head extracts of supplementary reproductives had a stimulating effect upon supplementary reproductive development when fed to orphaned groups of pseudergates (Lüscher, 1956). It is possible that these head extracts contained brain hormone.

If the brain hormone theory is correct, we must suppose that the pheromones of the reproductives normally inhibit the release of brain hormone, but that it is released suddenly and in large quantities in competent pseudergates when the pheromones are lacking. It is supposed that a massive release of brain hormone simultaneously activates the prothoracic glands and acts upon other tissues, thus inducing supplementary reproductive differentiation, which also involves a switchover of corpora allata function from production of juvenile hormone to production of gonadotropic hormone (see schematic representation in FIGURE 5).

Soldier Differentiation

The first stage of soldier development is the molt of the white soldier or presoldier, which always molts into a soldier 11 to 17 days later.

Presoldier molts occur rarely and irregularly in laboratory cultures. In nature they seem to occur chiefly in late spring, when a great number of nymphs undergo their final molt to become adults.

Presoldier molts can be induced experimentally by transplanting corpora allata of freshly molted supplementary reproductives into pseudergates (Lüscher, 1958). If the treated termites are kept in normal colonies, most of them will molt into presoldiers after 35 to 40 days. In orphaned colonies differentia-

tion is accelerated and most presoldier molts occur 20 to 25 days after the operation (Lüscher and Springhetti, 1960). The gut is generally emptied seven days before the molt, that is, the molt itself is induced eight to nine days before molting or some 15 or 30 days after the operation, according to the composition of the colony. Thus the implanted corpora allata induce presoldier differentiation, but the differentiation process is accelerated when the pheromones of the reproductives are absent. Lack of pheromones, according to the

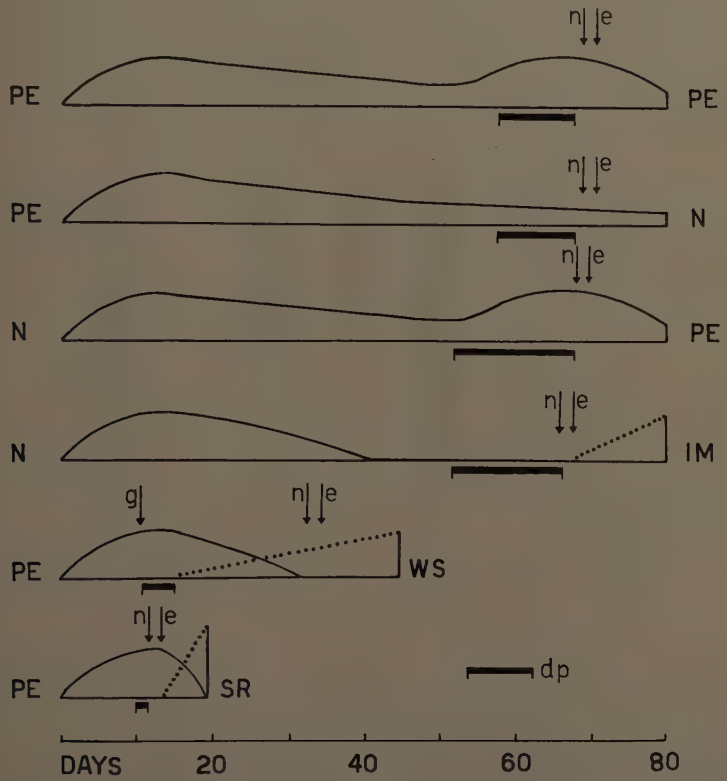

FIGURE 5. The supposed hormone actions in caste differentiation in *Kalotermes flavicollis*. Abscissa: age within the molting interval. PE = pseudergate; N = nymph; IM = imago (adult); WS = white soldier; SR = supplementary reproductive; n = neurosecretion; e = ecdyson (prothoracic gland hormone); g = gonadotropic hormone. The continuous curve indicates the supposed juvenile hormone titer; the broken curve indicates production of the gonadotropic hormone of the corpora allata; dp = determination period.

hypothesis put forward above, means a stimulation of neurosecretion that might accelerate differentiation, eventually, by stimulating proteosynthesis (Thomsen and Møller, 1959). The release of brain hormone, in the beginning, must be insufficient for an effective stimulation of the prothoracic gland, which seems to come about only when differentiation can be completed. This follows from the fact that intermediate forms between soldiers and pseudergates were very rarely produced in our experiments, but could be produced readily by injecting ecdyson ten days after the operation. Some intermediate forms between supplementary reproductives and presoldiers arise in orphaned col-

onies, where they can be explained as the result of an induction of supplementary reproductive development occurring before the termination of presoldier differentiation. In this case, as in the case of nymphal and adult development, we must assume that differentiation sets in some time before molting is induced, and that the timing of the molting process is controlled by a mechanism that comes into action when a certain state of differentiation is reached.

A comparative study of the effect of implanted corpora allata from different donors revealed remarkable differences. The results of these experiments, which were all performed in orphaned colonies, are shown in FIGURE 6. No

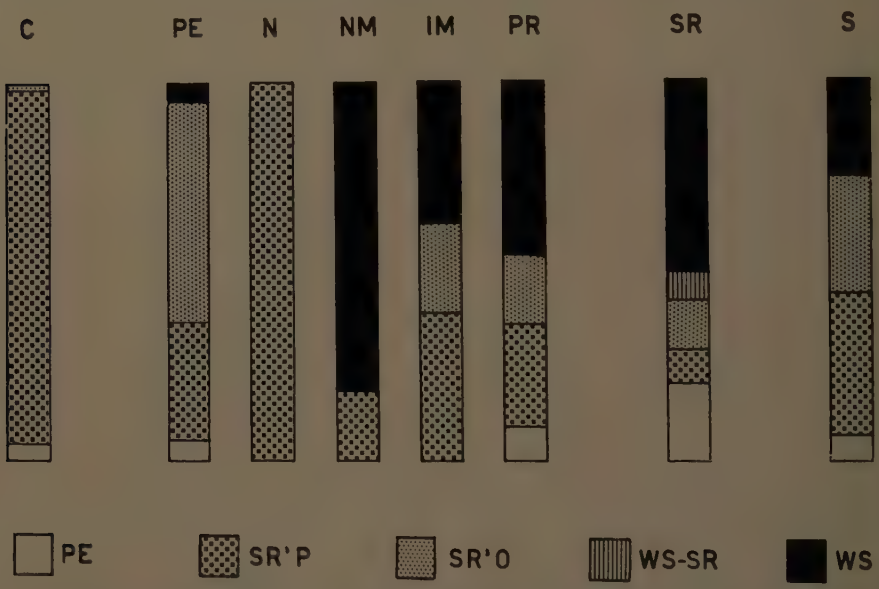

FIGURE 6. Percentage of castes of molted individuals after implantation of corpora allata from different donors. The donors are indicated on top of each column. *Donors:* C = controls; PE = pseudergates; N = second stage nymphs in middle of intermolt period; NM = second stage nymphs before adult molt; IM = alates (winged imago); PR = primary reproductives; SR = supplementary reproductives; S = soldiers. *Resulting castes:* PE = pseudergate; SR'P = supplementary reproductives with pigmented eyes; SR'O = supplementary reproductives without pigmented eyes; WS-SR = intercastes between white soldiers and supplementary reproductives; WS = white soldiers.

soldiers were produced after implantation of corpora allata from second-stage nymphs taken in the middle of the intermolt period. This is not astonishing, since a cessation of juvenile hormone production is very probable at this stage. However, corpora allata from pseudergates, in which they certainly produce juvenile hormone, also did not induce soldier differentiation, although two pairs of glands were transplanted in many cases. Alternatively, a high proportion of presoldiers were produced when corpora allata from supplementary reproductives, alates, or primary reproductives taken from incipient colonies were transplanted. In all these cases the corpora allata may be expected to produce gonadotropic hormone in the donor organism. This indicates that

the corpora allata produce two different hormones, which have different effects on caste differentiation.

Another observation made during these experiments leads to the same conclusion. The eyes of supplementary reproductives are normally pigmented, while larvae, pseudergates, and nymphs have no visible eyes. Eye pigmentation therefore is an adultoid character. The corpora allata of pseudergates, which were unable to induce soldier differentiation after transplantation, had an inhibiting action upon eye pigmentation in the supplementary reproductives that were produced in large numbers; most of them had completely unpigmented eyes. This is obviously a juvenile hormone effect. As can be expected, the corpora allata from second-stage nymphs, which do not produce juvenile hormone, had no such effect, but those taken from nymphs preparing for the adult molt induced soldier development. It therefore cannot be the action of juvenile hormone that induces soldier differentiation. It is more probable that this is the effect of the gonadotropic hormone. According to recent investigations on the respiratory metabolism of *Leucophaea*, the gonadotropic hormone may be identical with the metabolism-stimulating hormone of the corpora allata, but these two are probably not identical with juvenile hormone (Sägesser, 1960). Soldier differentiation may therefore be an effect of a general stimulation of metabolism.

It may be seen from FIGURE 6 that in those experiments in which presoldiers were produced and in which we therefore assume a gonadotropic function of the implants, there was at the same time a juvenile effect with an inhibition of eye pigmentation in supplementary reproductives. It seems probable therefore that the implants may switch over to some extent in the production of juvenile hormone under the influence of the larval hormonal milieu. It is also possible that the corpora allata of reproductives produce some juvenile hormone besides gonadotropic hormone. It is interesting to note that this cannot be true for the corpora allata of second-stage nymphs, which seem to produce gonadotropic hormone towards the end of the nymphal stage, but will not switch over to juvenile hormone secretion after transplantation.

If we are right in assuming that the gonadotropic hormone is responsible for inducing soldier differentiation, we have to postulate also that the corpora allata of soldiers produce gonadotropic hormone, since transplantation of these into pseudergates resulted also in presoldier development. If this is true, why do not the soldiers become fertile and produce eggs? It may be the *raison d'être* of the prothoracic glands in soldiers to prevent this. The prothoracic glands do not degenerate in soldiers as they do in other adults, although soldiers will never molt again. Engelmann (1959) has shown that implanted prothoracic glands prevent egg development in the adult female of *Leucophaea* by an inhibition of the corpora allata and possibly also by a direct action upon the ovaries. If the prothoracic glands are responsible for the infertility of soldiers in termites, the rare occurrence of fertile soldiers, which has been described for different species, may be due to an insufficiency of the prothoracic glands.

As we have seen, it is probable that the gonadotropic hormone of the corpora allata initiates soldier differentiation. Now the question arises: Is gonadotropic hormone taken up from outside, or is a switchover in the function of the corpora allata brought about by some unknown external influence? The

fact that presoldiers are produced in nature chiefly at the time when many nymphs molt into adults suggests the possibility that these nymphs, whose corpora allata already produce gonadotropic hormone, may give off some of the hormone with their excreta, which are normally immediately taken up by the pseudergates. It is also possible that the first larva that develops in an incipient colony and generally soon becomes a presoldier gets some gonadotropic hormone from its parents in this way. Of course the possibility of this transfer of hormone remains to be proved, and the question mentioned above cannot be decided at this time.

The Reacting System and Its Competence for Differentiation

The capacity of the reacting material to differentiate in different directions is inherent to this system; the hormones probably are doing no more than regulating and controlling the realization of the potentialities latent in the

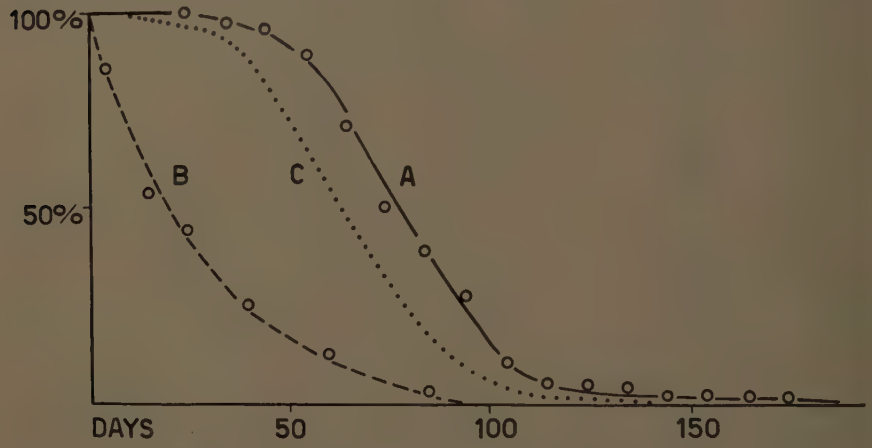

FIGURE 7. Competence curves. For explanation, see text.

"substrate." The substrate changes in the course of development as a whole, but it also changes during each molting cycle. The competence of a pseudergate for supplementary reproductive differentiation is highest just after the molt. It then decreases gradually and is finally completely lost towards the end of the intermolt period. Curve A in FIGURE 7 shows the percentage of pseudergates of different age within the molting interval that have not molted in normal colonies. Curve B shows the percentage of pseudergates of different age that will change into supplementary reproductives if the colony is orphaned and if the newly formed reproductives are not removed from the colony. In that case the colony is deprived of pheromones for six to ten days. If the newly formed reproductives are removed, and the colony therefore remains deprived of pheromones for a longer time, more pseudergates will change into supplementary reproductives and a differentiation frequency corresponding to curve C in FIGURE 7 results. This curve also corresponds to the presoldier differentiation frequency, when corpora allata of supplementary reproductives have been implanted. If these operations are carried out in orphaned colonies, in which

all molted individuals are at once removed, we find a supplementary reproductive frequency corresponding to curve B and a presoldier frequency corresponding to the difference between curve C and curve B. Under these circumstances the pseudergates with the highest competence for changing differentiate at once in supplementary reproductive direction; those with a lower competence differentiate in soldier direction. With the onset of differentiation the direction of differentiation is determined and is generally changed no more. Only rarely are intercastes produced. There are always some individuals that cannot be forced into soldier or supplementary reproductive differentiation. They molt into pseudergates again. In these individuals the competence for differentiation must have been lost when the experiment was started.

It is clear from the curves in FIGURE 7 that there is a great variation in the time of loss of competence, as there is great variation in the duration of the molting interval of the pseudergates. On the whole the curves indicate that the very high competence for changing in the beginning of the intermolt period begins to decrease about 55 days before the next molt, and that the competence is completely lost at least 15 days before each pseudergate molt.

The gradual change in competence during the intermolt period is probably due to metabolic changes within the cells, but the juvenile hormone titer seems to be of some importance for the changing competence. As we have seen, the volume of the corpora allata reaches two maxima during the pseudergate intermolt period. These two maxima probably correspond to two phases of juvenile hormone secretion, of which the second is responsible for preventing nymphal development. The first maximum is reached when differentiation competence is very high. It may, therefore, have a significance for the competence. If this is true, we should expect an increase of competence after the implantation of juvenile corpora allata. In fact the transplantation of corpora allata of pseudergates into pseudergates resulted in a stimulation of supplementary reproductive production (85 per cent in experimentals, 64 per cent in control series), but the actual figures are yet too low to be statistically significant ($P = 0.05$). They suggest, however, together with the histological findings, that the juvenile hormone titer may be correlated to the competence for differentiation during the first part of each molting interval.

Conclusions

The experimental evidence at the present time is not sufficient to permit a conclusive account of the hormonal control of caste differentiation in termites, although it can be stated with certainty that caste differentiation is controlled by hormones. However, basing ourselves on the evidence and the arguments discussed in this paper, we may put forth the following hypothesis, the principles of which are shown in FIGURE 5.

After each pseudergate molt the corpora allata become active and secrete juvenile hormone, which brings the individual into a reactive state of competence for differentiation. If during this time the pheromones, which normally inhibit neurosecretion, are absent, a massive release of brain hormone brings about supplementary reproductive differentiation, triggering at the same time the prothoracic glands that secrete the molting hormone and causing

the corpora allata to switch over to gonadotropic hormone secretion. If, alternatively, during the same period gonadotropic hormone is taken up orally (trophallaxis), presoldier differentiation is initiated. This is also connected with a switchover of the corpora allata to gonadotropic function and, later, with a release of brain hormone and molting hormone when differentiation is almost terminated. Presoldier differentiation may be accelerated if some brain hormone and, eventually, some prothoracic gland hormone is released constantly under the influence of pheromone absence. If nothing happens during the competence period, further development is dependent upon the juvenile hormone titer during the second part of the intermolt period, and this again seems to be dependent upon nutritional influences. Where nutrition is good, the second activation of the corpora allata is prevented and a nymphal molt ensues. In instances of less effective nutrition, the corpora allata are activated some time before the molt and a stationary molt results. In nymphs of the second stage the same causes lead to more drastic differences in the juvenile hormone secretion and, accordingly, to regressive molts or, following complete inactivation of the corpora allata, to the differentiation of adults.

Although much work is still needed for analysis of the details that are, for the most part, not well understood at present, it is on the whole clear that the time, the relative duration, and the intensity of secretion of the different endocrine glands bring about differentiation in one or the other direction. Thus caste differentiation in termites, like metamorphosis, is controlled chiefly by a mechanism of differential timing of hormone secretions. In termites metamorphosis is only a special case of caste differentiation. Wigglesworth's idea of considering metamorphosis as a special case of polymorphism is therefore entirely justified.

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HORMONAL CONTROL OF MOLTING BEHAVIOR AND SCALE DEVELOPMENT IN INSECTS*

Hans Piepho

Zoological Institute, University of Göttingen, Göttingen, West German Federal Republic

It is by now well established that the morphological pattern that characterizes the various stages in the life cycle of lepidoptera depends upon the relative proportion in which the hormones ecdyson and the juvenile hormone are present in the blood of the animal. Ecdyson in the presence of juvenile hormone induces a larval molt; in the presence of less juvenile hormone, a pupal molt; and in the presence of little or no juvenile hormone, an imaginal molt.

Let me clarify here the fact that I understand by the word "molt" or "molting" not only the shedding of the old skin but also the entirety of developmental processes that culminate in casting off the old cuticle. Thus metamorphosis in lepidoptera consists of two molts: a pupal and an imaginal molt.

It is a rather simple matter to produce experimentally abnormal humoral systems in the postembryonic developmental stages of moths. For instance, if one removes the corpora allata of young waxmoth (*Galleria mellonella* L.) caterpillars, or increases the number of corpora allata in last instar caterpillars, one can obtain hormonal systems intermediate between those that normally cause a larval or pupal molt. Under the influence of such abnormal humoral systems, the molting caterpillars become intermediate forms, that is, creatures possessing both larval and pupal characteristics. As a matter of fact, it is possible to create in this manner a series of intermediates that range in character all the way from purely larval to purely pupal forms.[1]

Humoral systems intermediate between those that produce a normal pupal or imaginal molt may also be obtained. This can be achieved by increasing experimentally the number of corpora allata in young pupae. Such pupae then develop into creatures that possess both pupal and imaginal characters. And again it is possible by appropriate experimentation to obtain a series of forms that range from normal pupae through all grades of intermediates to normal adults.[1]

Molting Behavior

The waxmoth caterpillar spins a long, thin silk tube near the mid-wall of the honeycomb. Within this tube the animal lives and feeds. Here it can move freely and quickly, thus safeguarding itself from attack by bees.

When the humoral system, calling for a larval molt, is set into motion and a larval molt approaches, the caterpillar spins a molting cocoon. This cocoon is also a tube, but stronger than the one described above, longer than the body of the caterpillar, and open on both ends. Within this cocoon, the caterpillar undergoes its larval molt.

Quite a different type of cocoon is spun when, under the influence of the appropriate humoral system, the last-stage caterpillar prepares for the pupal

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molt. This cocoon is short, very strong, and an almost ovoid tube. At first this tube is closed at both ends, but before pupating the animal bites off one end, thus creating a flap through which the moth later emerges.

Some years ago, I became interested in ascertaining what kind of cocoon caterpillars that later become intermediates, that is, animals with partly larval and partly pupal characters, would spin. To this end, I once again produced abnormal humoral systems by implanting additional corpora allata into the body cavity of last instar caterpillars. The operated animals, I found, spun intermediate cocoons with characteristics of both cocoon types. Within these cocoons, the larvae then changed into intermediate forms. There was a clear correlation between the morphological features of these intermediates and the type of cocoons they produced. The evidence thus suggests that a specifically composed humoral system not only controls the morphological features but also guides the behavior pattern of these animals.[2,3]

Recently, my co-workers and I have begun to investigate further the problem of hormonal control of behavior in the wax moth. Our main question was: Is a full grown last-stage caterpillar, preparing its pupal cocoon, bound to this behavior—or can it be forced into spinning a larval cocoon when supplied with large amounts of juvenile hormone? We took caterpillars from cocoons that could be clearly identified as pupal, but which were not yet completed. Then we transplanted up to 30 corpora allata into the body cavity of each animal. The preliminary results of these experiments revealed the following: some of the caterpillars never spun another cocoon; others spun a second cocoon, but molted before the type of cocoon could be identified; still others spun a more or less typical pupal cocoon and, at molting, became intermediates, that is, possessed of larval as well as pupal features. In these latter animals it was found that their behavior and the morphological characters they developed after molting did not harmonize.

To explain these facts, one may postulate the existence of two critical periods. During the first period the behavior pattern is determined by hormonal control; during the second, determination of the morphological features takes place. Thus if the humoral condition of the animal is changed experimentally after the first period has passed, the behavior pattern remains unaffected, while the morphological characters may still be altered. However, it must be emphasized again here that further experimentation is needed to verify these assumptions.

The investigator interested in the problem of hormone-controlled behavior in insects has much material available for study. For instance, let us consider dragonflies. The insect undergoes its larval molts at the bottom of pools. When the imaginal molt approaches, the animal becomes negative geotactic or positive phototactic, or both, leaves the pool, and molts. Ephemeridae exhibit much the same behavior. Caterpillars of cabbage butterflies molt at the food plant near the ground. Before they molt into a pupa, they become negative geotactic, leave the plant, crawl up walls or trees, and pupate high above the ground. On the other hand, some hawk moth caterpillars live and molt high in the tops of trees or bushes. With the approaching pupal molt, these animals become positive geotactic, crawl down from their high living quarters, and pupate in the ground.

Recently my associates and I experimented with the Linden hawk moth (*Mimas tiliae* L.).[4] These caterpillars undergo four and sometimes five larval molts. All larval molts occur at the feeding place at the top of linden trees. When a larval molt approaches, the caterpillar takes on a characteristic posture by stiffening its body and holding onto a twig only by its hind legs. In this position it finally sheds its old skin. It never spins a cocoon during the larval molting process. Drastically different is the behavior of the last stage caterpillar preparing for the pupal molt. It becomes positive geotactic, perhaps also negative phototactic and chemotactic (the behavior traits are not yet fully understood), crawls down the trunk of the linden tree, and digs into the ground. Here it spins a very loose cocoon and pupates.

The aim of our investigation was to ascertain what kind of behavior would be exhibited by a hawk moth in which the humoral system was intermediate between that calling for a larval or a pupal molt. We foresaw several possibilities:

(1) The hawk moth caterpillars would become intermediates in behavior, like the wax moth caterpillars described above. Perhaps they would stay halfway up the tree trunk, spin an incomplete cocoon and molt. The distance the caterpillars descended the tree trunk could be a measure of the juvenile hormone titer in their blood. Thus one could perhaps measure the juvenile hormone concentration by inches.

(2) The caterpillars might switch from one type of molting behavior to the other without intermediate behavior stages.

(3) This second possibility leads directly to the third. Granted, they can only express their behavior in two patterns; certain intermediate hormone systems may call for larval and others for pupal molting behavior. It would be interesting to discover which humoral conditions elicit one or the other response.

In order to produce experimentally the desired abnormal humoral systems, we had to transplant thousands of corpora allata. Since it was impossible for us to rear so many hawk moth caterpillars, we transplanted corpora allata from wax moths to hawk moths. One may recall that, years ago, I demonstrated that the action of the corpus allatum hormone was not species-, genera-, family-, nor even order-specific.[5] Today we know that crabs, worms, and other lower animals also contain substances with juvenile hormone activity.[10]

Since the hawk moth is a very large animal in comparison with the wax moth, we transplanted up to 24 wax moth corpora allata into hawk moth caterpillars. At the time of the operation, the hosts were in the first half of their last instar. The morphological effect of adding juvenile hormone to these hawk moth hosts was the same as that in the wax moth. We obtained an unbroken series of intermediates, ranging from normal larvae to normal pupae.

FIGURE 1*a* illustrates an animal that is almost a normal pupa. The only larval feature is a spot of larval cuticle (LC) at the site where the corpora allata were placed into the body. This phenomenon, as I have demonstrated earlier, is caused by the high sensitivity of the regenerated wound epidermis to juvenile hormone, and is not the result of a localized high allatum titer from the transplant near the wound.[5] One will also notice in this photograph that the old larval skin (C) was not completely removed from the pupa. The hormone

FIGURE 1. A graded series of Linden hawk moth intermediates, ranging from an almost normal pupal (*a*) to an almost normal larval individual (*f*). LC = spot of larval cuticle; C = cuticle of last larval instar (for further explanation, see text).

system that induced molting in this animal must have been very similar to one that induces a normal pupal molt, for this individual is an almost normal pupa. Caterpillars that produce pupae of this type were found to behave like normal animals that were about to undergo a pupal molt. They left their feeding place, crawled down the tree, dug into the ground, spun a loose pupal cocoon, and pupated.

FIGURE 1*b* is a photograph of an individual with somewhat more larval features than the foregoing one. Most of its cuticle is a hard, dark brown pupal cuticle. The antennae, mouth parts, wings, and thoracic legs are almost pupal in character. But larval pro-legs, although atypical in shape, and a small larval horn are present. The humoral system that induced the molt of this animal must have contained juvenile hormone in higher concentration than the foregoing individual. Before this caterpillar molted, it behaved as if it were performing a normal larval molt. It stayed up in the tree, clutched a twig with its hind legs, and exhibited the characteristic stiff posture of an animal in larval molt. However, shortly before this creature cast off its cuticle, the atypical hind legs could not hold the heavy body on the twig and the animal fell to the ground. Yet it did not try to dig in.

FIGURE 1*c*, *d*, *e*, and *f* illustrates a series of four individuals in which each specimen is a little more larval-like. Therefore, each of the molt-inducing hormone systems must have contained a somewhat higher allatum hormone titer respectively. These four animals exhibited, before they molted, typical larval molting behavior.

The results obtained in the wax moth and the hawk moth may be interpreted as follows. In the wax moth, the structural as well as the behavioristic features are apparently equally sensitive to intermediate hormone systems and respond in concert. Under the impact of such abnormal hormone systems, the caterpillars become morphological intermediates between larva and pupa and express a behavior pattern that also is composed of larval and pupal characteristics.

Developmentally, the hawk moth caterpillars respond to intermediate hormone systems, as do the wax moth specimens, by the formation of morphological intermediates. Behavioristically, however, their response to such hormone systems is different, in that they do not show intermediate behavior patterns. They either exhibit a larval or a pupal molting behavior. Animals in which the larval characters dominate always behave as if they were to undergo a normal larval molt, while intermediates that have predominantly pupal features show pupal molting behavior.

Scale Development

The developmental steps that lead to the formation of bristles and scales in lepidoptera are well known. Kruminš[7] investigated the development of bristles in the embryonic skin of the wax moth. His account of these events may be summarized briefly as follows. First, large cells appear among the normal epidermis cells. Each of the former divides; their mitotic spindles are oriented vertically to the surface. Then the upper of these two daughter cells divides again; the spindles are now oriented at an angle of about 45°

towards the surface. By these two so-called differential mitoses, a group of three cells is produced. From one of these, the bristles proper originate. The second forms the bristle socket and the third gives rise to a sensory cell. Now the development of scales and follicles during the metamorphosis of butterflies proceeds in much the same way. Only the third cell, that is destined to become the sensory cell, dies. Therefore these organs have no sense cells.

Follicles and scales of the same type as are found in butterflies also exist in the lowest insects. The sugar or silver mite *Lepisma saccharina* L. (Thysanoptera) hatches from the egg without scales and follicles and does not develop these organs in the following two molts. The first scales and follicles appear after the third molt. At each successive molt thereafter, the old scales and follicles are shed and new ones are produced. In addition to this, at each molt new scales and follicles develop at the spaces between the already existing ones. Thus at each molt, save for the first and second, the silver mite adds new scales and follicles to its skin.[8] E. M. Schmidt, in a recent paper,[9] stated that the early events (differential mitoses) of follicle and scale formation take place in the embryonic ectoderm, while these organs actually appear after the third molt. We have not been able to confirm this.

In the light of the foregoing considerations, two main problems emerge: (1) Are the molts of such primitive insects as *Lepisma* also controlled by hormones? (2) If they are, must we then assume that one hormone system induces a molt without, and another a molt with, scale and follicle formation?[8] To settle this issue experimentally, we employed a method that we have used successfully in our earlier work on caterpillars. We simply cut a piece of skin from a caterpillar and transplant it into the body cavity of another individual. Floating freely in the body cavity, the transplant survives and develops in a characteristic manner. The epidermis grows out from the edges of the cut surfaces and moves over the cuticle, thus forming a closed vesicle. When, under the influence of its humoral trigger, the host molts, the transplant molts simultaneously, performing the same type of molt as its host.

A slight variation of this method was used for our *Lepisma* studies. We took first instar silver mites that had just hatched from the egg, cut off their heads and tails, and implanted the remaining part into the body cavity of adult silver mites. The transplants survived, became vesicles, and molted at the same time as their hosts. During this molt, new scales and follicles developed, not only in the epidermis of the host, but also in the epidermis of the transplanted piece. The transplant, in other words, underwent the same type of molt as its host and thereby developed scales and follicles precociously. FIGURE 2 shows a section through such a molted transplant. One will notice that the first instar cuticle is shed into the interior of the vesicle: it contains no scales or follicles. Closely attached to the epidermis one may see the new cuticle, which contains scales and follicles. If this piece of skin had remained in its donor, it would have produced scales and follicles only after two more molts.

The results of these experiments are clear. Molting in *Lepisma* is under humoral control. Apparently two different hormone systems are available. One system, operating in the first and second instar, induces molting without

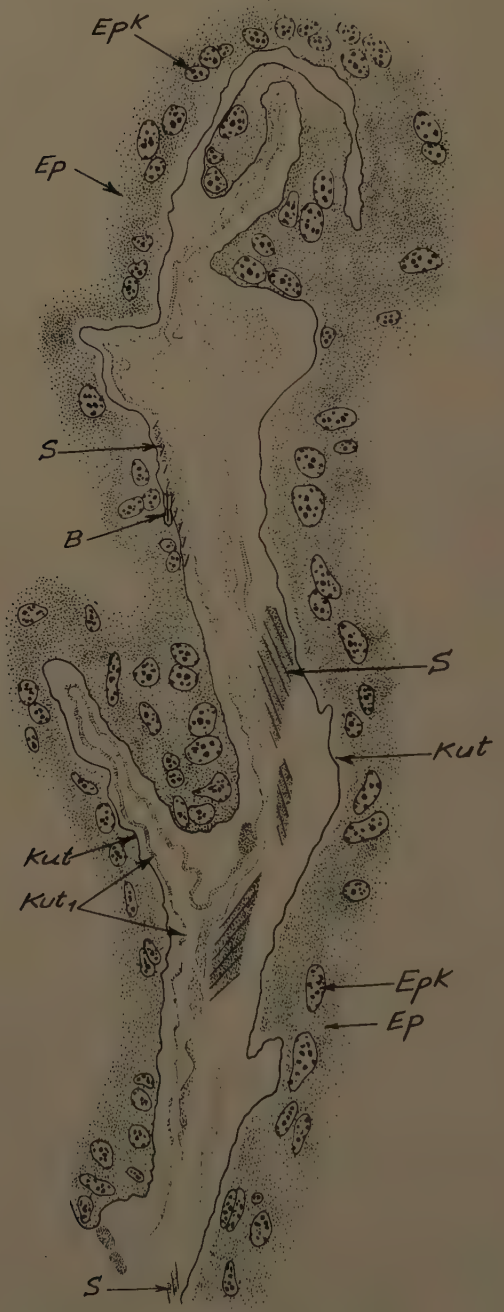

FIGURE 2. Section through a transplanted piece of first instar *Lepisma* skin after its first postoperative molt. B = scale follicle; Ep = epidermis; Epk = nucleus of an epidermis cell; Kut_1 = first instar cuticle without scale and scale follicles; Kut = epidermis with new cuticle, showing scales and scale follicles; S = scale (for further explanation, see text).

the formation of scales and follicles. The other system induces molts that are always associated with the development of scales and follicles. This system functions in the third and in all following instars.

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